

Oxygen sensor nanoparticles for monitoring bacterial growth and characterization of dose–response functions in microfluidic screenings

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Received: 15 April 2014 / Accepted: 18 August 2014 / Published online: 26 August 2014
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Abstract We are presenting a microfluidic droplet-based system for non-invasive, simultaneous optical monitoring of oxygen during bacterial cultivation in nL-sized droplets using ~350 nm nanobeads made from polystyrene and doped with the NIR-emitting oxygen probe platinum (II) 5, 10, 15, 20-*meso*-tetraphenyltetraabenzoporphyrin (PtTPTBP). Data were readout by a two-channel micro flow-through fluorimeter and a two-channel micro flow-through photometer. The time-resolved miniaturized optical multi endpoint detection was applied to simultaneously sense dissolved oxygen, cellular autofluorescence, and cell density in nL-sized segments. Two bacterial strains were studied that are resistant to heavy metal ions, viz. *Streptomyces acidiscabies* E13 and *Psychrobacillus psychrodurans* UrPLO1. The study has two main features in that it demonstrates (a) the possibility to monitor the changes in oxygen partial pressure during metabolic activity of different bacterial cultures inside droplets, and (b) the efficiency of droplet-based microfluidic techniques along with multi-parameter optical sensing for highly resolved microtoxicological screenings in aquatic systems.

Keywords Droplet-based microfluidics · Oxygen sensor · Optical probe-doped micro and nanoparticles · Micro cultivation · Highly-resolved dose/response function

Introduction

The increasing importance of the role of bacteria and other microorganisms in nearly all aspects of life comes in for the need of an extensive characterization of bacterial growth conditions and interactions among different bacteria and with other organisms. This interplay is important for health and biotechnology, but also required urgently for insights into complex processes in our environment, soil protection and agriculture, such as development of forests, lakes, rivers and oceans. It is finally crucial for supporting the growth of mankind, prosperity of industry, sustainable economy and the development of climate and biosphere on our planet [1, 2].

Next to the various processes microorganisms play a key role, since microorganisms are spread universally over all biotopes and are highly diverse in their properties, physiological and ecological behaviour [3]. This evokes in a high number of different proteins stored in the genomes of all microorganisms on earth, which enables the existence of very different populations and in very extreme environments [4, 5]. Up to now, it is impossible to detect all types of microorganisms and to characterize their properties and individual behavior. The huge number of different types and the large spectrum of environmental conditions and effectors prevent not only the extensive understanding, but is also a serious threshold for solving of the most essential problems in connections with microorganisms. It is evident that the dilemma of large numbers for investigation and control of microorganisms cannot be solved by conventional techniques.

The success in the development of new methods to investigate microorganisms and their interplay is the adaptation of

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the scientific and technical methods to the dimensions and principles of these organisms themselves. A first progress was already achieved with the improvement of the microscopic, trace analytics and molecular biological techniques [6–8]. With it an enhanced sensitivity for the read-out of molecular data from small cells or even from single molecules was realized. The various and complex relations between organisms and their environment are, however, still subject of numerous investigations. Thus, suitable methods have to cope with the dimensions, volumes and spatial organization of life of microorganisms and populations. For these reasons, microfluidic technologies offer very promising solutions in terms of compartmentalization, manipulation and sensing of liquids on a very small scale [9]. Among them, the droplet-based techniques and micro segmented flow techniques are particular powerful. These techniques allow the reduction of sample volume down to the nanoliter and picoliter range which enables a large number of separate investigations and the processing of hundred to millions of liquid compartments in parallel. Furthermore, fluid segments are simple to manipulate and arrange in well-ordered sequences applying different microfluidic operations [10, 11].

Miniaturized optical techniques have been introduced for the non-contact read-out of cellular growth inside the micro fluid segments. In particular, micro flow-through photometry and fluorimetry are well suited for the measurement in micro fluid segments and are used for the screening of dose/response relations in microtoxicological investigations. These optical techniques enable the characterization of bacteria responses towards toxic substances in detail as well as the identification of combinatorial effects by two- and three-dimensional concentration screenings [12].

Despite the high potential of these applied methods, the specificity of the information read-out from cell populations inside micro fluid segments is limited when measuring simply cell density and autofluorescence utilizing photometry and fluorimetry. Novel automated methods for the read-out of chemical information are sought after in order to get more detailed information about the on-going physiological processes inside these small droplets. In principle, many specific molecular assays, e.g. immunoassays [13] and nucleic acid amplification-based assays [14]—have been demonstrated in microfluidics and read-out by optical procedures. However, the sensitive and reproducible implementation of automated microtoxicological screenings is still a formidable challenge.

In a previous study we accomplished pH determination using sensor microparticles for monitoring of bacterial growth inside micro fluid segments, whereby dose/response functions could be determined [15]. Changes in pH are indicative of the physiological activity of cells by aerobic dissimilation or fermentation and supply complementary information to optical density measurements.

Oxygen is another immensely important chemical species in almost all living organisms [16]. Therefore the analytical chemistry of oxygen plays a major role in biology. The realization of the large variety of analytical tasks with biological samples of different types and characteristics requires a range of analytical methodologies, sensor materials, measurement instrumentation and accessory tools. Oxygen can be measured by chemical (Winkler titration [17]), or electroanalytical (Clark-type electrode [18]) methods and optical oxygen sensors. Optical techniques become more and more attractive due to numerous advantages such as non-invasive measurements, continuous monitoring, in situ analyses, option of miniaturization, biocompatibility, reversibility, precision and accuracy. Regarding different optical techniques, the phosphorescence quenching-based oxygen sensor [19] has evolved as a powerful analytical method with a large range of applications. This sensor produces a characteristic response as a result of a photophysical (phosphorescence quenching) process that is linked to the oxygen concentration of the sample. It can be determined in a non-contact procedure measuring the luminescence intensities, radiometric intensities or lifetimes of dedicated probe-doped sensor layers, micro- or nanoparticles [20–24]. Optical oxygen respirometry using phosphorescence was applied for determining the growth kinetics of microorganism [25, 26] and the analysis of various effectors on the growth of different aerobic bacteria, aquatic organisms and mouse embryos [27–29].

It is known that many *Streptomyces* and *Bacillus* species produce an amount of secondary metabolites which can be found in various medical applications [30]. The effect of heavy metal ions on microorganisms is essential for the evaluation of their role in natural biosystems. High concentrations of heavy metals are known to cause oxidative stress and damage organisms [31]. We investigated the applicability of oxygen nanosensor particles PS-PtTPTBP for monitoring the growth of two heavy metal resistant bacterial strains *Streptomyces acidiscabies* E13 and *Psychrobacillus psychrodurans* UrPLO1 in micro fluid segments and their potential for microtoxicological investigations on copper ions.

Experimental

Chemicals

Perfluoromethyldecaline (PP9) was obtained from F2 Chemicals (www.f2chemicals.com). $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ was from Merck (www.merck.de). Phosphate buffered saline (PBS) solution was composed of 137 mM NaCl, 2.68 mM KCl, 10.14 mM Na_2HPO_4 , 1.76 mM KH_2PO_4 .

Oatmeal agar for *Streptomyces acidiscabies* E13 cultivation was consisted of 1.5 % oatmeal and 2 % agar. M3 medium for *Streptomyces acidiscabies* E13 incubation was

consisted of 25.5 mM glucose monohydrate, 2.5 % soluble starch, 1.0 % peptone from casein, 0.5 % yeast extract, 11.35 mM $(\text{NH}_4)_2\text{SO}_4$, 11.02 mM KH_2PO_4 , 49.96 mM CaCO_3 and 1 mL trace element solution. The trace element solution was composed of 0.29 mM ZnCl_2 , 0.74 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 41.23 μM $\text{CuCl}_2 \cdot 6\text{H}_2\text{O}$, 50.53 μM $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 8.33 μM $(\text{NH}_4)_2\text{Mo}_7\text{O}_{24} \cdot 6\text{H}_2\text{O}$ and 49.75 μM $\text{Na}_2\text{B}_4\text{O}_7$.

AM minimal medium for *Streptomyces acidiscabies* E13 and *Psychrobacillus psychrodurans* UrPLO1 for pre-cultivation and micro screening experiment contained 3.78 mmol asparagine, 2.87 mM K_2HPO_4 , 81.14 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 35.97 μM $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 50.46 mM glucose monohydrate.

Test organisms and cultivation

Strain *Streptomyces acidiscabies* E13 is deposited at the Jena Microbial Resource Collection Center (JMRC, WDCM 919) with the accession number JMRC:FSU:E13 *Streptomyces acidiscabies*. The strain E13 was grown on oatmeal agar. For the pre-cultivation, the bacteria E13 was transferred from agar plates into 10 mL M3 medium. The strain E13 was cultivated for 24 h at 28 °C at 120 rpm. From these pre-cultures containing vegetative mycelium, 0.1 mL suspension was evenly spread on oatmeal agar plates. After 10–14 days of cultivation at 28 °C, conidiospores from aerial hyphae were harvested using 5 mL sterile PBS solution per plate. In order to remove excess mycelium, the suspension was centrifuged at 1,000 rpm for 2 min. Afterwards the spore containing supernatant liquid was used for the experiments. The spores were diluted with AM minimal medium to obtain 10^7 spores·mL⁻¹. This spore suspension was used for inoculation of separated droplets in the microfluidic system.

Psychrobacillus psychrodurans strain UrPLO1 was obtained from Friedrich-Schiller-University Jena, Institute of Microbiology, Department of Microbial Communication. The strain UrPLO1 was grown on AM agar [32]. For the pre-cultivation a single colony of UrPLO1 was transferred from an agar plate into an Erlenmeyer flask filled with 10 mL AM minimal medium, and cultivated for 24 h at 28 °C under constant stirring. For the microfluidic experiments a cell suspension with a density of about 10^7 cells·mL⁻¹ was applied.

Oxygen-sensitive nanobeads

Internal oxygen consumption during bacterial growth was investigated using microspheres PS-PtTPTBP containing ca. 2 % (w/w) platinum (II) 5, 10, 15, 20-*meso*-tetraphenyltetraenzoporphyrin in polystyrene [33, 34] in polystyrene (PS-PtTPTBP). PtTPTBP was purchased from Frontier Scientific (www.frontiersci.com). Styrene and $\text{K}_2\text{S}_2\text{O}_8$ were from Sigma-Aldrich (www.sigmaaldrich.com),

methanol and chloroform from VWR (www.vwr.com), Visking dialysis membranes from Roth (www.carloth.com) and paper filters from Whatman (www.gelifesciences.com), dd H₂O was produced in-house.

Polystyrene nanoparticles were produced according to the following procedure: 5 g styrene was mixed with 36 g dd H₂O in a 10 mL round flask equipped with a reflux condenser and temperature control. 0.1 g $\text{K}_2\text{S}_2\text{O}_8$ was dissolved in 9 g dd H₂O and immediately added to the styrene/water mixture. It was then heated to 95 °C under reflux for 4 h. After cooling to room temperature, the particle suspension was dialyzed for several days against dd H₂O using Visking cellulose membranes and filtrated using a cellulose paper filter. 2 mL methanol, 1 mL ddH₂O and 500 μL of the nanoparticle suspension of the previous step were stirred for 15 min in a small glass beaker. 25 μL chloroform was added and the mixture was stirred for 10 min. The stirbar was removed and 1 mg PtTPTBP dissolved in 500 μL chloroform was added and the mixture was shaken for 4 h. Afterwards a gentle nitrogen stream was bubbled through the beaker for 30 min and the suspension was filtrated in a glass pasteur pipette filled with glass wool. The filtrate was dialyzed for several days against dd H₂O using Visking cellulose membranes and filtrated using a cellulose paper filter. Mean particle hydrodynamic diameter was determined to be 357 ± 53 nm via dynamic light scattering (RiNa, www.rina-gmbh.eu). Optical spectra were determined on an AB2 spectrometer (Thermo, Idstein, Germany) and a MonoVista microspectrometer (S & I, www.s-and-i.eu). This material demonstrated good long-term photostability and biocompatibility. Optical properties and oxygen sensitivities of the particles in PBS are displayed in Fig. 1. The sensor particles have excitation maxima at 430 nm and 614 nm. The emission maximum is located at 778 nm. PS-PtTPTBP particles feature a strong dependence of their emission

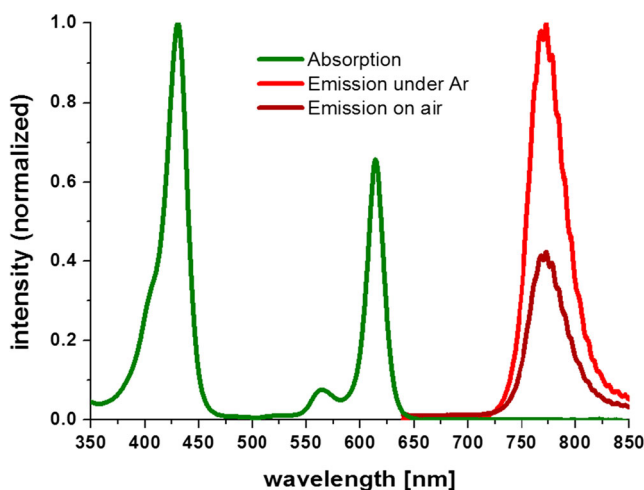


Fig. 1 Optical properties of employed oxygen sensor nanoparticles, 50 ppm (w/w) in PBS, pH 7.4. Absorption of PtTPTBP (green line) and emission of the PS particles in argon (red line) and air atmosphere (brown line)

intensities on oxygen in aqueous suspensions as evident from the spectra recorded in PBS, 150 mM, pH 7.4 in the presence and absence of atmospheric oxygen content.

The PS-PtTPTBP sensor particles were calibrated with a two-point calibration at 0 % and 100 % air saturation (Table 1). The zero content was checked by immersing the sensor particles into a 0.08 M sodium sulfite solution. The oxygen concentration and phosphorescence intensity was determined with a multi-channel micro fiber optic oxygen transmitter (PreSens OXY-4) and a microflow-through fluorimeter at room temperature (23.8 °C).

The nanobeads PS-PtTPTBP was used to monitor the growth of an oxygen-consuming *Psychrobacillus psychrodurans* UrPLO1 and *Streptomyces acidiscabies* E13 bacterial cultures inside segments.

Microfluidic arrangement and on-line measurements

Details on the fluidic devices, the optical detector, the generation and analysis of segments for the microfluidic screening experiments used herein were reported in detail previously [12]. In the present work, an experimental set-up related to the previous work and shown schematically in Fig. 2a was used. Four syringes with a volume of 500 µL, each including effector, beads suspension, cultivation medium and cell suspension and one syringe with a volume of 5 mL for the carrier liquid were mounted on a multiaxis computer-controlled syringe pump system (Cavro Centris, www.tecan.com). The syringes were connected by PTFE tubing with an inner diameter of 0.5 mm and an outer diameter of 1.6 mm (www.bohlender.de) to a PEEK 7-port manifold (www.upchurch.com). The generation of micro fluid segments was realized through this manifold by controlled dosing of effectors, cultivation medium, beads suspension and cell suspension into a constant flow of carrier liquid PP9.

Generated segments are transported with a constant flow rate via transparent FEP (fluorinated ethylene propylene) tubing (ID 0.5 mm, OD 1.6 mm) through an optical detection unit containing a flow-through photometer and a fluorimeter (Fig. 2b). Two light-emitting diodes (www.agilent.com) with peak wavelengths of 470 nm and 505 nm were used for light scattering measurements during bacterial growth and to monitor the segment size, distance between two neighbouring segments and the segment number. With these parameters the determination of segment loss or fusion due to long cultivation time or repeated measurement processes can be executed for error correction. For monitoring cell-derived

autofluorescence of the heavy metal-tolerant bacteria *Streptomyces acidiscabies* E13 and *Psychrobacillus psychrodurans* UrPLO1 an LED (www.cml-it.com) with a peak wavelength of 470 nm with a combination of a shortpass excitation (<480 nm) and a longpass emission filters (>510 nm) was used (www.lasercomponents.com). For phosphorescence intensity recording of oxygen sensor particles inside micro fluid segments a laser diode with a peak wavelength of 405 nm (www.cnilaser.com) with a combination of a shortpass (<445 nm) and longpass filters (>650 nm) was used (www.lasercomponents.com). The emitted photons were detected by photomultiplier modules (www.hamamatsu.com). The normalized fluorescence intensity value I_n (unitless) is given by the ratio of the intensity of the measurement value I_{flu} to the reference fluorescence I_{ref} of the FEP tube filled with carrier medium subtracted by 1.

$$I_n = \frac{I_{flu}}{I_{ref}} - 1$$

The tube coils consisted of a PMMA plate with rolled PTFE tubes with a length of seven meters (ID 0.5 mm, OD 1.6 mm) that were used to store and to incubate the generated segments. To investigate growth kinetics and dose–response effects on bacteria, all segments were moved inside one tube coil through the detector unit to a second storage tube coil after a certain “static” incubation time. Therefore, this technique allows multiple runs without regenerating segments.

Experimental procedure

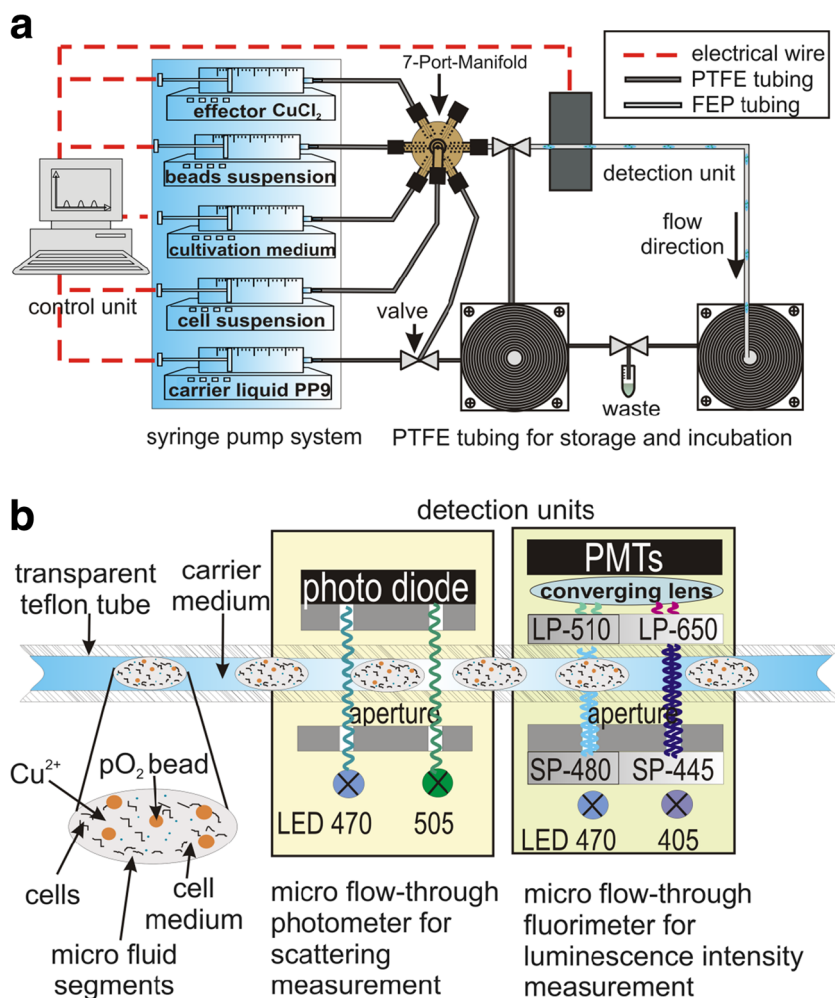
To investigate the dose–response relationships for CuCl₂ on *Psychrobacillus psychrodurans* UrPLO1 a LabView program with continuous change of the effector concentration was used. The flow rates of the carrier liquid PP9 and the cell suspension were set at 40 µL·min⁻¹ and 7.5 µL·min⁻¹, respectively. The flow rates of the effector solution and the cultivation medium were varied depending on the concentration combinations, but were adjusted to a total flow rate of 7.5 µL·min⁻¹. Thus, the overall flow rate of the segment generation process was kept constant at 55 µL·min⁻¹. An initial cell density of about 10⁷ cells·mL⁻¹ corresponding to about 2,000 cells per segment was applied. The average size of segments is 400 nL and the length of the spacers is 2.2 mm (PTFE tube ID 0.5 mm). The final concentration of Cu²⁺ was 1.0 mM.

In total, we analyzed about 50 segments for every measurement point for the cultivation experiments independence of

Table 1 Two-point calibration for 0 and 100 % air saturation at room temperature

Measurement method	pO ₂ at 100 % a.s.	pO ₂ at 0 % a.s.
PreSens OXY-4	7.8 mg·L ⁻¹	0 mg·L ⁻¹
Microflow-through fluorimeter normalized phosphorescence intensity	0.50±0.05	1.05±0.05

Fig. 2 Microfluidic set-up and arrangement of applied optical sensors for micro droplet sequences through the tube. **a** Model of the experimental set-up. **b** Scheme of the optical detector units with two channel micro flow-through photometry and fluorimetry for endpoint detection of bacterial growth, endogenous autofluorescence intensity and phosphorescence intensity inside micro fluid segments



incubation time with and without influence of pO_2 -beads and about 600 segments for the microtoxicological screening with Cu^{2+} on *Psychrobacillus psychrodurans* UrPLO1. This redundancy was applied in order to prove measurement reliability and to exclude or identify stochastic effects eventually caused by the small reaction volume and the small number of cells for incubation. After formation and data acquisition the segments were directly transported into tube coils, thus a serialized analysis was possible. Thereafter the tube coils were stored for up to 3 days of incubation at 28 °C for remeasurement.

Results and discussions

Monitoring of growth kinetics of *streptomyces acidiscabies* E13 in micro fluid segments

In our previous study, we could demonstrate that the micro fluid segment technique is applicable for the cultivation and screening using the heavy metal-tolerant actinobacterium *Streptomyces acidiscabies* E13 as a target organism [35].

The present study targets on the investigation of growth kinetics and therefore on oxygen consumption applying PS-PtTPTBP oxygen sensing beads cultivated with *Str. acidiscabies* E13. Each segment was inoculated with about 2,000 spores. The growth and the oxygen consumption were evaluated at many different time points by determination the autofluorescence intensity at 470 nm excitation (emission at >510 nm) and the phosphorescence intensity at 405 nm excitation (emission at >650 nm). Growth was observed 20 h, 32 h, 45 h and 67 h after the start of the incubation (Fig. 3a). The continuous increase of fluorescence intensity with excitation at 470 nm over the incubation time reflects the growth of bacterial cells during the incubation (Fig. 3a). In contrast to the emission in the autofluorescence channel (emission at >510 nm), there was nearly no emission in the oxygen channel (emission at >650 nm). But if 250 $mg \cdot L^{-1}$ PS-PtTPTBP oxygen sensitive particles were added to the cultivation liquid, the low emission at 650 nm (Fig. 3b) was enhanced during the bacterial growth inside the fluid segments, in analogy to the autofluorescence increase at 510 nm during the first 24 h. This enhancement was caused by consumption of oxygen of the

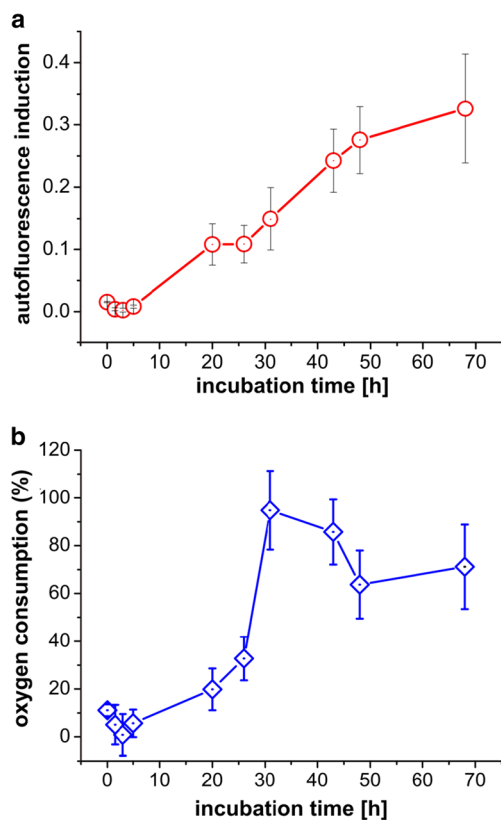


Fig. 3 Monitoring of autofluorescence and phosphorescence intensity of *Str. acidiscabies* E13 cultivated inside micro fluid segments. **a** Autofluorescence intensity determined by in situ micro flow-through fluorimetry (excitation: 470 nm, emission: >510 nm) at different cultivation time points. **b** Segment internal pO₂ development determined by phosphorescence intensity read-out of oxygen sensor particles PS-PtTPTBP (excitation: 405 nm and emission: 650 nm)

growing amount of bacteria relying on aerobic metabolism. The limited diffusion of oxygen through the tube walls was not able to completely compensate the oxygen content. After 24 h incubation time, a stronger increase of phosphorescence was observed reflecting a significant rise of the metabolic activity. The high phosphorescence of the sensor beads corresponded to a drastic reduction of the oxygen concentration and was due to the increased cell number and their high oxygen consumption. After 48 h incubation time, a certain decrease of phosphorescence was observed reflecting higher phosphorescence quenching of the sensor particles by oxygen. This decrease could be interpreted by a reduction of metabolic activity and a partial compensation of consumed oxygen inside the fluid segments by the slow diffusion from outside through the tube wall.

Monitoring of growth kinetics of *psychrobacillus psychrodurans* UrPLO1 in micro fluid segments

In these experiments bacterial culture *Psychrobacillus psychrodurans* strain UrPLO1 in the lag phase and the early

exponential phase of bacterial growth were used as inoculum culture. At the beginning about 600 micro fluid segments of 400–450 nL were generated for the investigation of growth kinetics and oxygen change inside segments with application of PS-PtTPTBP beads cultivated with *Psychrobacillus psychrodurans* UrPLO1. The number of cells per fluid segment was kept constant and inoculated with about 2,000 cells per segment. The growth and oxygen kinetics were evaluated at nine different cultivation time points by measuring the cell density using micro flow-through photometry with a peak wavelength of 470 nm, cellular autofluorescence intensity at 470/>510 nm as well as phosphorescence intensity of pO₂ sensing beads at 405/>650 nm using micro flow-through fluorimetry.

All three sensor signals reflected the fast growth of the bacterial cultures inside the fluid segments during the first 20 h. But certain differences in the information supplied by the different optical channels could be obtained. The steep increase of intensity loss by scattering (Fig. 4a) was followed by a slight decrease which indicates a slight enhancement of transmission. It is assumed that this signal indicates a termination of the bacterial growth phase after 20 h followed by a moderate change in the distribution of the cells (probably a partial aggregation) at the second and the third day of cultivation. The autofluorescence signal (Fig. 4b) confirmed the strong increase in cell number and metabolic activity of the growing population during the first day. But this increase continued during the second day. This behavior could be explained by an on-going intensification of metabolic activity connected with the production of fluorescing species after achieving the highest cell density. The autofluorescence signal at >650 nm only increased minimally during the cultivation in the absence of sensor particles (Fig. 4c black star line). When 250 mg·L⁻¹ PS-PtTPTBP oxygen-sensitive particles were added to the cultivation liquid the continuous high metabolic activity was also reflected in the O₂-sensor signal (Fig. 4c blue diamond line), the high phosphorescence at >650 nm indicated a low oxygen content and indicated against a reduction of oxygen consumption by the bacteria during the second and the third day of cultivation.

Characterization of effect of copper ions on metal-tolerant *bacillus* strains by monitoring the oxygen content inside the micro fluid segment

The advantages of pO₂ luminescent sensors particles, enabling contactless reliable sensing in nanoliter volumes were thus combined with multianalyte detection for microtoxicological applications. Therefore, linear programs providing a continuous increase of the model toxin Cu²⁺ in concentrations of 0–1 mM were applied in separate experimental screening runs.

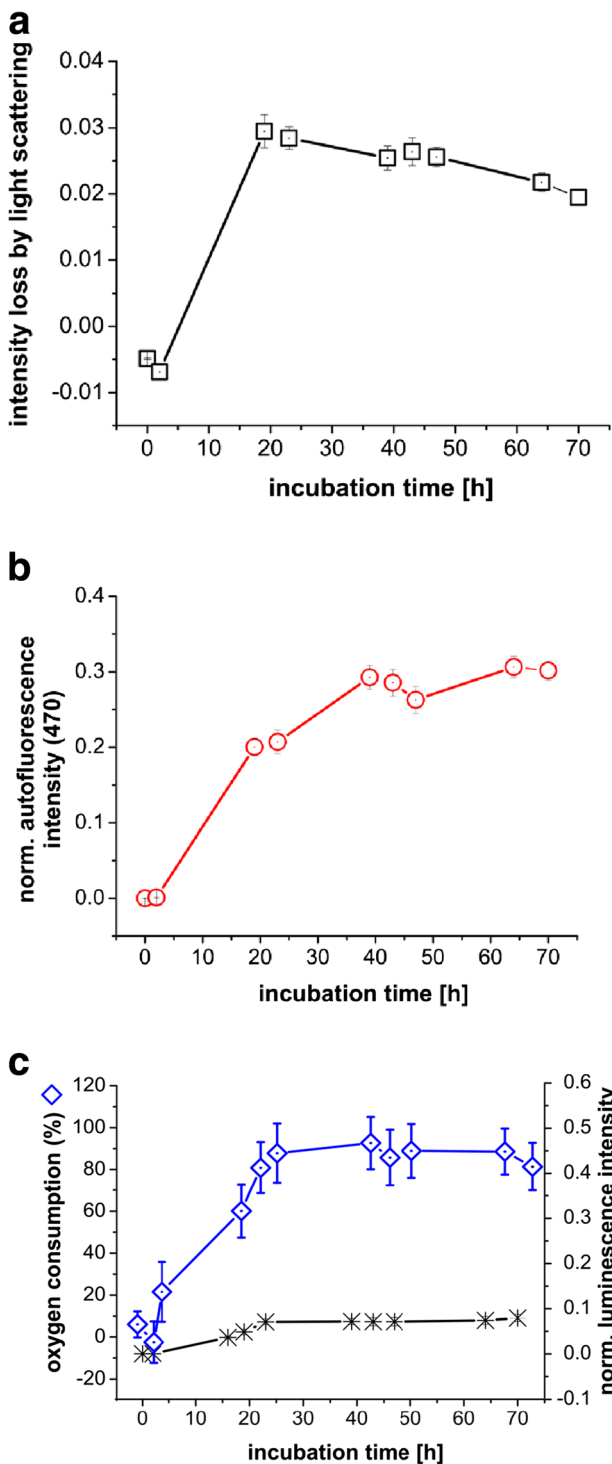


Fig. 4 Growth and metabolic activity monitoring of a *Psychrobacillus psychrodurans* UrPLO1 culture cultivated inside micro fluid segments. **a** Cell density determined by in situ micro flow-through photometry (470 nm) at different cultivation time points. **b** Autofluorescence intensity determined by in situ micro flow-through fluorimetry (excitation: 470 nm, emission: >510 nm). **c** Segment internal pO₂ development determined by fluorimetric read-out (excitation: 405 nm, emission: >650 nm) by using oxygen sensor particles PS-PtTPTBP (blue diamond line) and fluorescence intensity without sensor beads (black star line)

Nearly 600 micro fluid segments of 400 nL were generated. The change of segment internal cell density, autofluorescence

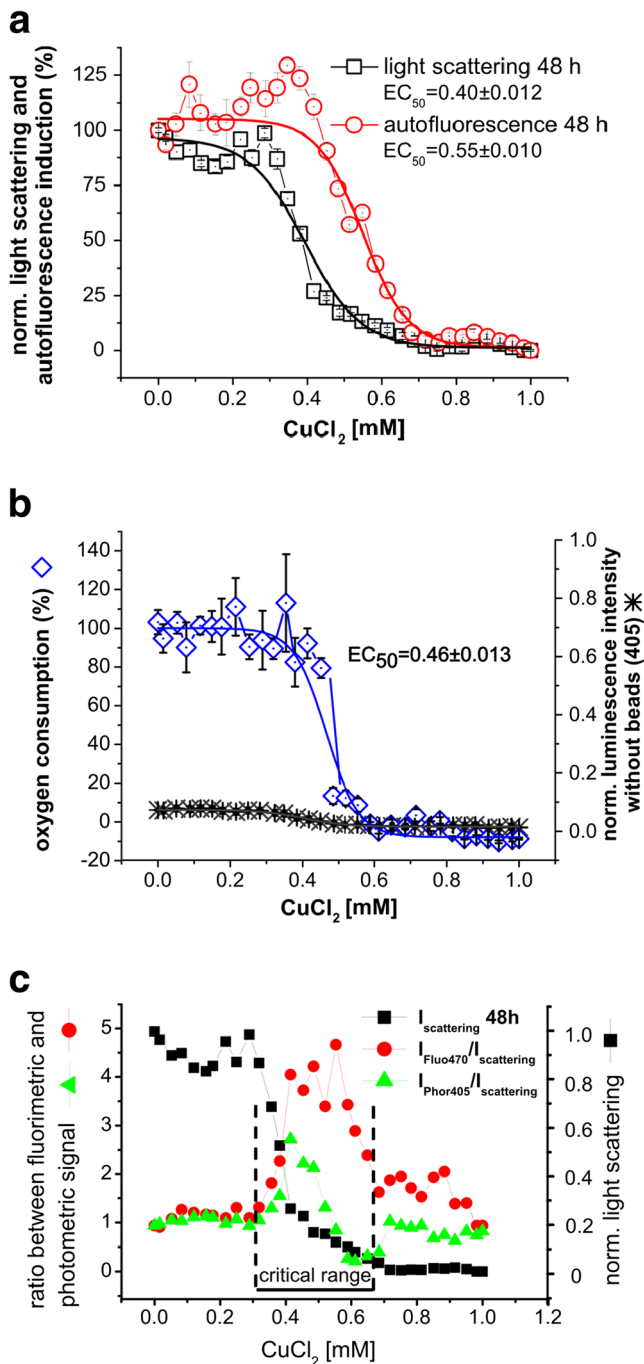


Fig. 5 Highly-resolved dose/response curves of *Psychrobacillus psychrodurans* UrPLO1 against CuCl₂ obtained in micro fluid segments after an incubation period of 48 h. **a** Circles: autofluorescence intensity determination using micro fluorimetry. Squares: intensity loss by light scattering measured by using micro photometry. **b** Monitoring of oxygen consumption inside the segments using oxygen sensor particles PS-PtTPTBP (diamond) and fluorescence intensity without sensor beads (star). **c** Ratios between fluorimetric and photometric signals

Table 2 Figures of merit of common oxygen optical detection for monitoring bacterial growth

Ref.	Method	Sensing scheme	Advantages	Limitations
Preininger et al. [36] Riedel et al. [37]	Technique of biological oxygen demand (BOD), biosensor arrangement using immobilized cells	Fluorescence intensity; thin film solid-state sensors	Faster response than BOD ₅ , possibility of in situ monitoring; easy calibration; low-cost disposable sensors	Large layer thickness leads to slow response and slow kinetics; recalibration after reconditioning
Papkovsky et al. [38] O'Mahony et al. [27, 29] Jasionek et al. [26]	Respirometric screening technology (RST) of microtiter plates	Quenching of phosphorescence by intensity or lifetime measurements, indicator dye water-soluble	Disposable oxygen sensor, real-time monitoring; high sensitivity; non-invasive sensing; cheap; high flexibility	Oxygen influx critical; evaporating; possibility of inhibition of samples; interference by sample fluorescence and turbidity
Stitt et al. [25]	BD oxygen biosensor system (OBS), integrated optical oxygen sensing in microtiter plates	Fluorescence intensity measurement; indicator dye embedded in polymer (layer thickness 200 μm)	Real-time readout; HTS compatible format; disposable OBS microtiter plates	Slow response and slow kinetics; oxygen influx critical; limited flexibility; evaporation effect
Arain et al. [28] and John et al. [39]	PreSens Oxoflate; integrated optical oxygen sensing in microtiter plates	Fluorescence intensity measurement; indicator dye embedded in hydrogel (layer thickness 10 μm)	High resolution and accuracy at lower oxygen concentration; fast response; easy calibration	Limited flexibility; evaporation effect
Lam et al. [40]	Microfluidic differential oxygenator system	Phosphorescence intensity measurement; thin film solid-state sensors (PtOEPK in PS)	Real-time oxygen sensing, set repeatable DO concentration, robust and low-cost chip	Individual calibration of every DO sensor; limited flexibility;
This work	Droplet based-microfluid technique with microflow through fluorimeter	Phosphorescence intensity measurement; ptfPTBP in PS nanoparticles	Nanoliter volume; non evaporation; in situ, non-invasive and real-time measurements; HTS screening; high flexibility	Possibility of inhibition of sample; oxygen influx critical; interference by sample fluorescence and turbidity

and dissolved oxygen were evaluated after 48 h by using micro flow-through photometry and fluorimetry.

On first sight, all three signals displayed in Fig. 5a and b seemed to reflect nearly the same toxic effect of the copper ions on the bacteria. However, a more detailed analysis of the transition region between high bacterial growth (below 0.30 mM Cu²⁺) and the growth inhibition above 0.70 mM provides evidence that these three sensor signals supply different information about this transition region.

The obtained dose/response functions measured by autofluorescence showed intense growth in all segments containing up to 0.50 mM Cu. The EC₅₀ value, defined as the concentration which causes 50 % reduction in autofluorescence intensity of *Psychrobacillus psychrodurans* UrPLO1 compared with the controls, was determined to be about 0.55 mM (Fig. 5a circles). In light scattering detection an EC₅₀ value of 0.40 mM was determined (Fig. 5a squares). The autofluorescence relative to the scattering intensity was stronger in the transition region. It can be concluded that in this region the bacteria are growing slower but showing much higher metabolic activity per cell. This can be due to an increase in fluorescing primary metabolites but could even be an indication of the enhanced induction of a fluorescent secondary metabolite in the critical concentration range. The different character of scattering and autofluorescence signal led to a more or less parallel shift of the dose/response curve from about 0.40 mM up to about 0.55 mM. The decline of both signals was considerable, but the decline observed in the concentration dependence of the oxygen sensor beads signal was much steeper in direct comparison (Fig. 5b diamond). A drastic reduction in the oxygen consumption in a very small concentration interval between about 0.46 and 0.48 mM Cu²⁺ was observed.

From the comparison of the three curves (ratios between fluorimetric and photometric signal), it can be concluded that the oxygen consumption of *Psychrobacillus psychrodurans* UrPLO1 in the sub lethal range between about 0.3 and 0.5 mM Cu²⁺ was enhanced and the enhancement correlates with the increase of absolute autofluorescence in this range. Between concentrations of 0.5 and 0.7 mM Cu²⁺, the bacterial growth was significantly reduced but not completely inhibited. In this range the oxygen consumption was strongly reduced, but the autofluorescence per cell is still high which indicates a strong stimulation of production of fluorescing species by the bacteria in this concentration range (Fig. 5c).

The results show the phosphorescence intensity measurement produced by the oxygen sensor particles at the recommended working dilution were sufficient for the oxygen sensing inside segments. However, a more robust and accurate technique to quantify oxygen is using miniaturized luminescence lifetime-based oxygen sensor instrumentation which will be implemented in future work. Compared to the other common biological oxygen optical detection systems offers

microfluidic technique for monitoring bacterial growth and toxicological evaluation an attractive method but still has room for further development (Table 2).

Conclusion

In conclusion, our study demonstrates that simultaneous nanoparticle-based phosphorescence sensing of the oxygen along with photometric and autofluorescence mapping in micro fluid segments could be performed using NIR-emitting metallobenzoporphyrin-doped polystyrene beads added to the culture liquid. The phosphorescence signal of applied sensor particles was not influenced by the minimal medium and did not show detectable losses caused through bleaching effects or particle decay over the complete cultivation period up to 72 h. The results with application of oxygen sensor particles are in good agreement to the results from light scattering and autofluorescence measurement for the growth kinetic experiments with two different microorganisms.

We also showed the technique of micro segmented cultivation and in situ growth monitoring with application of oxygen-sensitive nanoparticles is well suited for the determination of highly-resolved dose/response functions for individual effectors. The microtoxicological investigation of copper(II)chloride on a soil bacterium *Psychrobacillus psychrodurans* UrPLO1 using PS-PtTPTBP nanobeads confirmed its pronounced tolerance against Cu²⁺. The simultaneous registration of signals from three optical channels allowed identification of the critical concentrations of heavy metal ions by different responses from the grown bacteria populations. The investigated example showed the possibility of extraction of differential information on the metabolic activity of cells by separate interpretation of the three registered signals by cell density (reduction of photometric signal by light scattering), general aerobic metabolic activity (phosphorescence intensity of oxygen sensitive particles) and production of fluorescing species (autofluorescence signal). Generally, the introduced method provided a rapid and easy tool for the investigation of growth and metabolic activities during the cell cultivation inside micro fluid segments.

Acknowledgments J. Cao is funded by German Federal Environmental Foundation under contract No. 20009/009. S. Nagl is funded by and thanks the German Research Foundation (DFG, NA 947/1-2). The authors gratefully acknowledge the funding from German Federal Ministry of Education and Research in the frame of the project BactoCat (Kz: 031A161A). We thank S. Schneider, F. Weise and F. Möller for excellent experimental and technical assistance, Prof. N. Sträter and Dr. C. Roth (Institute of Bioanalytical Chemistry, University of Leipzig) for dynamic light scattering and Dr. A. Funfak (Laboratoire d'Hydrodynamique, Ecole Polytechnique, Palaiseau, France) for helpful discussions.

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