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Effect of selenite on the morphology and respiratory activity of *Phanerochaete chrysosporium* biofilms

Erika J. Espinosa-Ortiz ^{a,*}, Yoan Pechaud ^b, Ellen Lauchnor ^c, Eldon R. Rene ^a, Robin Gerlach ^c,

Brent M. Peyton ^c, Eric D. van Hullebusch ^b, Piet N.L. Lens ^a

^a UNESCO-IHE Institute for Water Education, Westvest 7, 2611 AX Delft, The Netherlands

^b Université Paris-Est, Laboratoire Géomatériaux et Environnement (EA 4508), UPEM, 77454 Marne-la-Vallée, France

^c Center for Biofilm Engineering, Montana State University, 366 EPS, PO Box 173980, Bozeman, MT 59717, USA

HIGHLIGHTS

- Exposure of *Phanerochaete*

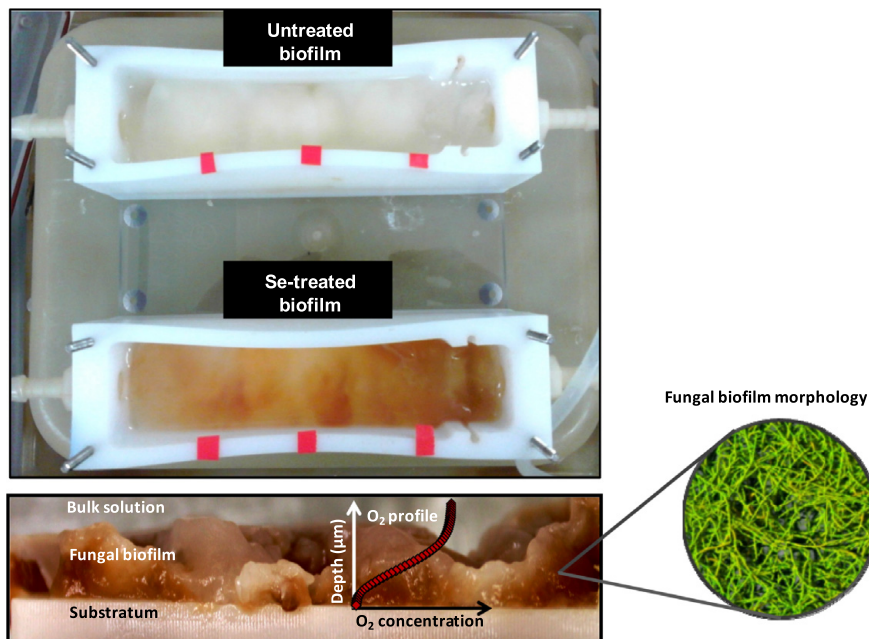
chrysosporium biofilms to SeO_3^{2-} was investigated.

- 20% decrease in O_2 flux occurred in

the biofilm upon 24 h of SeO_3^{2-} exposure.

- A more compact and less porous

hyphal biofilm structure was induced by SeO_3^{2-} .



ABSTRACT

KEYWORDS

Fungal biofilm
Phanerochaete
chrysosporium

Selenium

Oxygen profiles

Microelectrodes

The temporal and spatial effects of selenite (SeO_3^{2-}) on the physical properties and respiratory activity of *Phanerochaete chrysosporium* biofilms, grown in flow-cell reactors, were investigated using oxygen microensors and confocal laser scanning microscopy (CLSM) imaging. Exposure of the biofilm to a SeO_3^{2-} load of $1.67 \text{ mg Se L}^{-1} \text{ h}^{-1}$ (10 mg Se L^{-1} influent concentration), for 24 h, resulted in a 20% reduction of the O_2 flux, followed by a $\sim 10\%$ decrease in the glucose consumption rate. Long-term exposure (4 days) to SeO_3^{2-} influenced the architecture of the biofilm by creating a more compact and dense hyphal arrangement resulting in a decrease of biofilm thickness compared to fungal biofilms grown without SeO_3^{2-} . To the best of our knowledge, this is the first time that the effect of SeO_3^{2-} on the aerobic respiratory activity on fungal biofilms is described.

1. Introduction

Selenium (Se), an element belonging to group 16 of the periodic table (chalcogens), plays a dual role as an essential micronutrient at low concentrations, but it can also be toxic to organisms at higher concentrations (Lenz and Lens, 2009). An overdose of Se disrupts the integrity of proteins and decreases cellular enzymatic activity, leading to severe consequences for human health, including dermal, respiratory and neurological damage (USHHS, 2003). Se is of significant research interest from an environmental viewpoint due to its toxicity in natural water bodies. In particular, the water-soluble oxyanions selenite (SeO_3^{2-}) and selenate (SeO_4^{2-}), mainly related to agricultural activities, mining, chemical, textile, photographic and electronic industries, has shown to cause widespread impacts on aquatic life, leading to a cascade of detrimental bioaccumulation (Chasteen and Bentley, 2003; Lemly, 2004; Lenz and Lens, 2009).

The use of biological agents to remove pollutants from water can be an efficient, economic and environmentally friendly alternative to conventional physicochemical methods, e.g. chemical precipitation, catalytic reduction and ion exchange. Different microorganisms have the ability to convert SeO_3^{2-} and SeO_4^{2-} into insoluble forms, mainly elemental Se (Se^0). The biomineralization of Se has been used for bioremediation of soils and wastewater treatment, with the potential to recover Se biomaterials (Nanchaiah and Lens, 2015). Most of the reported biotechnologies for the treatment of Se polluted effluents are based on bacterial metabolism (Nanchaiah and Lens, 2015), although the use of fungi is also promising due to their ability to grow under acidic conditions, ease of handling and their inherent capacity to produce high amounts of enzymes. The latter property is of particular importance since the reduction of metal/metalloid ions and the formation of their elemental nanoparticles is attributed to an enzymatic reduction process (Zhang et al., 2011).

Uptake and volatilization are the main Se coping mechanisms reported for fungi (Gharieb et al., 1995). Some fungal strains, including *Alternaria alternata* (Sarkar et al., 2011), *Lentinula edodes* (Vetchinkina et al., 2013) and *Phanerochaete chrysosporium* (Espinosa-Ortiz et al., 2015), have demonstrated to biomineralize Se^0 in the nano-size range. These bio-nanomaterials possess improved properties compared to the bulk material, making them a desirable product for use in photocells, semiconductor rectifiers and medical applications (Beheshti et al., 2013). Among the different fungi tested, most of them have been identified as SeO_3^{2-} -reducing organisms, but the reduction of SeO_4^{2-} to Se^0 has only been achieved with the fungal extract of *A. alternata* (Sarkar et al., 2011).

Previous studies on Se-reducing fungal strains were mostly performed using dispersed filamentous or self-immobilized (pellets) cultures (Espinosa-Ortiz et al., 2015; Gharieb et al., 1995). However, the morphological and inhibitory effects of Se oxyanions on fungal biofilms have not yet been reported. Biofilms, as naturally occurring communities of microorganisms, play key roles in the biogeochemical cycling of toxic elements in aquatic systems (van Hullebusch et al., 2003). Biofilms are complex, heterogeneously structured microbial communities attached to a substratum and enclosed in a self-produced extracellular matrix (Flemming and Wingender, 2010). The main differences between attached and dispersed microbial growth arise from the physical structure, leading to different mass transfer patterns, and substrate and nutrient concentration gradients resulting in significant physiological and respiratory heterogeneity.

Biofilms have been suggested to be more productive and metabolically active than planktonic cells, or the dispersed mycelium in the case of fungi, due to high concentrations of active enzymes and extracellular proteins (Ramage et al., 2012). The response of biofilms to inhibitory compounds has been addressed

using a number of methods based on indicative targets, e.g. respiratory and enzymatic activities, cell growth and cell viability. The use of microsensors to estimate the respiratory activity (O_2 uptake changes) in biofilms exposed to inhibitors has been well established and it has been widely used as a method to assess toxicity and inhibition (Lauchnor and Semprini, 2013; Hou et al., 2014). Therefore, we targeted fungi in a biofilm mode of growth to observe their response to a specific inhibitor (SeO_3^{2-}).

In this paper, we report for the first time the effects of SeO_3^{2-} on *P. chrysosporium*, a Se-reducing organism (Espinosa-Ortiz et al., 2015), when grown as a biofilm in a flow-cell reactor. Experiments were performed first to assess the effects of SeO_3^{2-} exposure on developed biofilms (short-term experiments) and secondly to assess the influence of SeO_3^{2-} on biofilm development (long-term experiments). O_2 microsensor measurements and confocal laser scanning microscopy (CLSM) imaging were employed to visualize the temporal and spatial changes induced by the presence of SeO_3^{2-} . O_2 consumption rates (biofilm activity) and the physical properties of the biofilms, i.e. porosity and density, were then estimated based on the O_2 profiles. The changes in the biofilm architecture caused by SeO_3^{2-} were observed directly using CLSM.

2. Methods

2.1. Fungal strain and culturing conditions

The white-rot fungus *P. chrysosporium* (ATCC 24725) was used throughout the study. The fungal strain was cultured for 3 days at 37 °C on potato dextrose agar slants. Sub-cultures were prepared as required and maintained at 4 °C. A fungal spore suspension, used as the inoculum for subsequent biofilm cultures, was prepared by suspending the culture from one agar slant in 50 mL liquid medium in an Erlenmeyer flask. The liquid medium contained (g L^{-1}): glucose, 2.5; KH_2PO_4 , 2; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5; NH_4Cl , 0.1; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1; thiamine, 0.001; and 5 mL of a trace element solution as described by Tien and Kirk (1988). The initial pH was adjusted to 4.5 with 1 M HCl, and the medium was sterilized at 123 kPa and at 121 °C for 30 min.

2.2. Biofilm growth and exposure experiments

Biofilms of *P. chrysosporium* were cultivated for 4 days in flow-cell reactors (15 cm $\times$ 3.8 cm $\times$ 3.5 cm) run in parallel in the presence (Se-treated) or absence (untreated) of SeO_3^{2-} (Fig. S1, Supplementary Material). Each reactor had a working volume of 0.12 L and a substratum surface area of 57 cm². The reactors were filled with medium (2.5 g glucose L⁻¹, pH 4.5, T 30 °C) and the fungus was allowed to grow in batch conditions for one day to facilitate the attachment of the cells. The reactors were then supplied with influent at a constant flow rate ($Q_i = 0.02 \text{ L h}^{-1}$) for 3 days to attain a nominal hydraulic retention time (HRT_n) of 6 h. The effluent was recycled ($Q_r = 0.09 \text{ L h}^{-1}$, recirculation to influent ratio = 4.5:1) to provide well-mixed conditions in the reactor. Thus, an actual hydraulic retention time (HRT_a) of 1.1 h was maintained. The substrate loading rate was maintained constant at 0.08 g glucose L⁻¹ h⁻¹, while the Se influent concentrations was either 10 or 20 mg Se L⁻¹, corresponding to a loading rate of 1.67 or 3.3 mg Se L⁻¹ h⁻¹, respectively. The influent concentrations of glucose and Se reported were measured in the feed medium prior to being mixed in the inlet of the reactors with the recirculation flow (sampling port 1, see Fig. S1, Supplementary Material); these values were used to estimate the removal rates and efficiencies in the reactor (see Section 2.5). Biofilms were cultivated in closed reactors using atmospheric air as headspace. Whenever required, the reactors were opened for the measurement of O_2 profiles and

care was taken to minimize the potential of external contamination. Microscopic imaging revealed no indication of bacterial or archaeal growth in the flow-cell reactors.

Short- and long-term SeO_3^{2-} exposure experiments were performed to assess the spatial and temporal effects on the fungal biofilms by measuring the O_2 concentration profiles at different times and locations within the biofilms:

- (1) *Short-term SeO_3^{2-} exposure tests:* Untreated 4 day-old biofilms were exposed to SeO_3^{2-} (10 mg Se L^{-1} , $1.67 \text{ mg Se L}^{-1} \text{ h}^{-1}$) for 3 h and 24 h. The O_2 profiles were determined *in situ* before the biofilms were exposed to SeO_3^{2-} (untreated) and again after 3 h and 24 h of SeO_3^{2-} exposure. The results from this experiment were used to determine the reduction in O_2 consumption rate within the biofilm upon sudden SeO_3^{2-} exposure.
- (2) *Long-term SeO_3^{2-} exposure tests:* Biofilms were cultivated for 4 days either in the absence or presence of SeO_3^{2-} . The response of the biofilms to (a) low (10 mg Se L^{-1} , $1.67 \text{ mg Se L}^{-1} \text{ h}^{-1}$), and (b) high (20 mg Se L^{-1} , $3.3 \text{ mg Se L}^{-1} \text{ h}^{-1}$) influent concentrations was determined. The SeO_3^{2-} concentration was doubled in experiment 2b to delineate the influence of a different SeO_3^{2-} load on the biofilm. A physical characterization of these biofilms was performed with CLSM. O_2 profiles were measured within the biofilms at the end of the incubation period (4 days) of each experiment.

2.3. Microsensor measurements

Microsensors for O_2 measurements were used to determine the influence of SeO_3^{2-} on O_2 concentrations within the *P. chrysosporium* biofilms. O_2 profiles over the depth of the biofilm at three different locations in the bioreactor were measured using a Clark-type O_2 microsensor with a tip diameter of 8–12 μm (Unisense, Denmark), having a detection limit of $0.01 \text{ mg O}_2 \text{ L}^{-1}$. Measurements were made *in situ*, by maintaining the flow continuously in the bioreactors, at room temperature and under atmospheric O_2 conditions. Positioning of the microsensors was done using a micromanipulator fitted with a motor controller, and the sensor output data was collected online using the SensorTrace Pro software (Unisense, Denmark). O_2 profiles were acquired in triplicate at each location. The profiles obtained were normalized by dividing the O_2 concentration measured at depth z by the O_2 concentration in the bulk solution. The observation of similar O_2 profiles for each triplicate suggested that pseudo-steady-state O_2 uptake was reached. Based on the O_2 profiles, the location of the biofilm surface was determined according to the procedure outlined by Wäsche et al. (2002).

2.3.1. Estimation of diffusion coefficients

The effective diffusion coefficients of O_2 in the biofilms were estimated from the O_2 profiles. Obeying Fick's law, and considering one-dimensional diffusion gradients in the biofilm, the O_2 flux in the boundary layer between the bulk solution and the biofilm was estimated according to Eq. (1). The O_2 flux in the biofilm was determined using Eq. (2) (Lewandowski et al., 1991):

$$\text{Flux, } J_w (\text{mg cm}^{-2} \text{ s}^{-1}) = -D_w \left(\frac{dC}{dz} \right)_{\text{External}} \quad (1)$$

$$\text{Flux, } J_f (\text{mg cm}^{-2} \text{ s}^{-1}) = -D_f \left(\frac{dC}{dz} \right)_{\text{Internal}} \quad (2)$$

where D_w ($\text{cm}^2 \text{ s}^{-1}$) is the O_2 diffusion coefficient in water and D_f ($\text{cm}^2 \text{ s}^{-1}$) is the diffusion coefficient within the biofilm. dC/dz is

the gradient O_2 concentration measured with the microsensors at the boundary layer (*external*) or within the biofilm (*internal*).

Assuming the fluxes J_w and J_f are equal at the surface, the diffusion coefficient D_f can then be calculated according to Eq. (3) (Wanner et al., 2006):

$$D_f = D_w \left(\frac{dC}{dz} \right)_{\text{External}} / \left(\frac{dC}{dz} \right)_{\text{Internal}} \quad (3)$$

The relative diffusivity (f_D), which is defined as the relation between D_f and D_w , was estimated using Eq. (4):

$$D_f = f_D D_w \quad (4)$$

2.3.2. Estimation of biofilm physical properties based on diffusion coefficients and O_2 inhibition

Zhang and Bishop (1994) developed a random porous cluster model, assuming that the microorganisms are randomly located in a fraction of the available sites in the biofilm, avoiding multiple occupancies. Based on this model, the porosity of the biofilm (θ) can be calculated according to Eq. (5):

$$\theta = \sqrt[3]{f_D} \quad (5)$$

The density (ρ , kg m^{-3}) of the biofilm can also be approximately calculated based on its correlation to the relative diffusivity, f_D (Fan et al., 1990), according to Eq. (6). It should be noted that this correlation is based on the experimental data obtained for different types of biofilms (including fungi), substrates and temperatures.

$$\frac{D_f}{D_w} = 1 - \frac{0.43\rho^{0.92}}{11.19 + 0.27\rho^{0.99}} \quad (6)$$

The global inhibition, I_j (%), i.e. the reduction in O_2 flux into the biofilms, and thus O_2 consumption rate, in the presence of SeO_3^{2-} relative to the control biofilms, was estimated according to Eq. (7) (Zhou et al., 2011):

$$I_j = 1 - \left(\frac{J_{W\text{Se-Treated}}}{J_{W\text{Untreated}}} \right) \quad (7)$$

2.4. Biofilm sectioning and imaging

Samples from biofilms grown for 4 days in the absence or presence of SeO_3^{2-} (20 mg Se L^{-1} , $3.3 \text{ mg Se L}^{-1} \text{ h}^{-1}$) were chosen randomly and stained with the fluorescent probe FUN 1 (Molecular Probes), as previously described elsewhere (Villa et al., 2011). Briefly, the biofilms were stained by covering the biofilm samples with 30 μM FUN 1 in phosphate buffered saline (PBS) solution at 30 °C for 30 min in the dark. The biofilms were then rinsed with PBS solution. The stained samples were covered with optimum cutting temperature formulation (OCT, Tissue-Tek, USA) and frozen with dry ice. The frozen core was embedded in more OCT, and then sectioned at -20 °C using a Leica CM1850 cryostat. Cryo-sections (7 μm thick) were visualized with a Nikon Eclipse E800 microscope in transmission mode using differential interference contrast optics. Images were processed using the software MetaMorph (Molecular Devices, Downingtown, PA). Biofilm architecture of the stained biofilms was visualized using CLSM (Leica TCS-SP2 AOBs). The software Imaris (Bitplane Scientific Software, Zurich, Switzerland) was used for processing the images.

2.5. Analytical methods

Influent and effluent samples were collected twice per day and centrifuged at 4700 g for 15 min, and the supernatant was used for analysis. Glucose concentrations were determined using the dinitrosalicylic acid method (Miller, 1959) with D-glucose as the

standard. The total Se concentration was measured using ICP-MS (Agilent 7500ce) after preserving the samples with 5% HNO₃ (Espinosa-Ortiz et al., 2015).

2.6. Performance parameters of the flow-cell reactor

The performance of the flow-cell reactor was determined by calculating the glucose and Se removal rates according to Eq. (8), while the removal efficiency (E) was estimated using Eq. (9):

$$\text{Removal rate, } R_i(\text{mg L}^{-1} \text{ h}^{-1}) = \frac{Q[C_0 - C_t]}{V} \quad (8)$$

$$\text{Removal efficiency, } E_i(\%) = \frac{C_0 - C_t}{C_0} \quad (9)$$

where Q (L h⁻¹) is the influent flow rate, C_0 (mg L⁻¹) is the influent concentration, C_t (mg L⁻¹) is the effluent concentration measured at time t , and V (L) is the working volume of the reactor (0.120 L). The subscript i in the equations indicates either Se or glucose.

2.7. Statistical analysis

Substrate and Se removal rates by *P. chrysosporium* biofilms are reported in this study. Averages and standard deviations were calculated for the long term experiments when steady state conditions were attained over the 4 day period of incubation. The effect caused by the presence of SeO₃²⁻ on the E_i was evaluated by performing analysis of variance (ANOVA) at $P_{\text{value}} \leq 0.05$.

3. Results and discussion

3.1. Influence of short-term SeO₃²⁻ exposure on *P. chrysosporium* biofilm activity

Short-term experiments were performed to ascertain the temporal effects of SeO₃²⁻ on the *P. chrysosporium* biofilms, allowing to estimate the inhibition of the global O₂ consumption. Se-free biofilms were grown for 4 days and then exposed to SeO₃²⁻ (10 mg Se L⁻¹, 1.67 mg Se L⁻¹ h⁻¹), for 3 and 24 h. O₂ concentration profiles were determined at different locations in the biofilm, before and after exposure to SeO₃²⁻ (Fig. 1). The O₂ concentration in the bulk solution varied between 7.0 and 7.5 mg O₂ L⁻¹. Triplicates of the O₂ profiles were acquired; similar profiles were obtained during the measurements of each location (data not

shown), which suggested a pseudo-steady-state O₂ uptake by the biofilm.

Table 1 summarizes the parameters obtained from the O₂ profiles. Before and after SeO₃²⁻ exposure, O₂ concentrations reached levels below the detection limit in the *P. chrysosporium* biofilm indicating the presence of anoxic zones within the biofilm (Fig. 1). Differences in the O₂ profiles observed over time revealed the influence of SeO₃²⁻ on the O₂ flux into the biofilm. While the differences between I_j values observed were not statistically different ($P_{\text{value}} = 0.13$), a decrease in O₂ consumption rate of 11.5 (±6.5)% after SeO₃²⁻ exposure for 3 h and 20 (±5.3)% upon exposure for 24 h was observed. The rates of glucose consumption (R_{glucose}) were estimated to be 0.052, 0.049 and 0.047 g glucose L⁻¹ h⁻¹ after 0, 3 and 24 h of SeO₃²⁻ treatment, respectively, corresponding to a 6% and 10% decrease in R_{glucose} . The rates of selenium removal (R_{Se}) were calculated to be 0.17 mg Se L⁻¹ h⁻¹ after 3 h and 0.22 mg Se L⁻¹ h⁻¹ after 24 h, corresponding to E_{Se} values of 12% and 15.5%, respectively.

The inhibition of the O₂ respiration and the decrease in the R_{glucose} of the biofilm indicate that SeO₃²⁻ exposure is inhibitory to the activity of *P. chrysosporium*. Even though O₂ profiles varied across the length of the reactor, which was attributed to the heterogeneous nature of the biofilm, a clear inhibition in O₂ consumption was observed in the presence of SeO₃²⁻. I_j did not vary significantly over time, suggesting that the inhibitory response of *P. chrysosporium* occurs within 3 h of SeO₃²⁻ exposure. This agrees with observations by Zhou et al. (2011) who observed a 29% inhibition of O₂ consumption within 1 h of exposure of wastewater biofilms to Zn²⁺ (5 mg L⁻¹). Analogous responses were observed in aerobic wastewater biofilms exposed to ZnO nanoparticles (Hou et al., 2014), wherein the O₂ respiration activity in the outer layer of the biofilm was inhibited (27%) within 2 h of ZnO nanoparticles exposure (50 mg L⁻¹).

Walters et al. (2003) showed stronger inhibitory effects of antibiotics (10 µg mL⁻¹ of ciprofloxacin or tobramycin) on cells in the more oxygenated areas of *Pseudomonas aeruginosa* biofilms, whereas the bacteria in the anoxic zones were more resistant. This was attributed to low metabolic activity in the anoxic basal layer of the biofilm. Further investigations should be performed in order to determine the local metabolic activity of the *P. chrysosporium* biofilm. This could for instance be achieved by visualizing spatial patterns of protein synthetic activity within the biofilm using epifluorescence microscopy as demonstrated by Walters et al. (2003).

The estimation of the inhibitory effect based on differences in the O₂ flux and Fick's first law of diffusion (Eq. (7)) is commonly considered to be a good overall estimate of biofilm respiratory activity, although uncertainties in these estimates can be associated with: (i) the estimation of the boundary layer as there are different methods to determine its location (Wäsche et al., 2002), (ii) the fact that the O₂ flux not only depends on the microbial activity but also on the physical properties of the biofilm (possibly porosity and density), and (iii) the overestimation of the interfacial flux at the boundary layer as the biofilm might become compressed when inserting the microsensor into the biofilm (Lorenzen et al., 1995).

The inhibitory effect of SeO₃²⁻ on fungal growth has been previously described for unicellular, polymorphic and filamentous fungi (Gharieb et al., 1995; Espinosa-Ortiz et al., 2015). The response of *P. chrysosporium* biofilms to SeO₃²⁻ exposure was similar to the responses observed for *P. chrysosporium* pellets (Espinosa-Ortiz et al., 2015). When grown as pellets in the presence of SeO₃²⁻ (10 mg Se L⁻¹, 10 g glucose L⁻¹, pH 4.5, 30 °C, 12 days incubation), the biomass production of the fungus decreased by ~80% compared to Se-free incubations, while the glucose consumption was limited to ~30%.

An orange-red coloration developed in the biofilm over time during SeO₃²⁻ exposure (not shown). After 3 h, a visible coloration

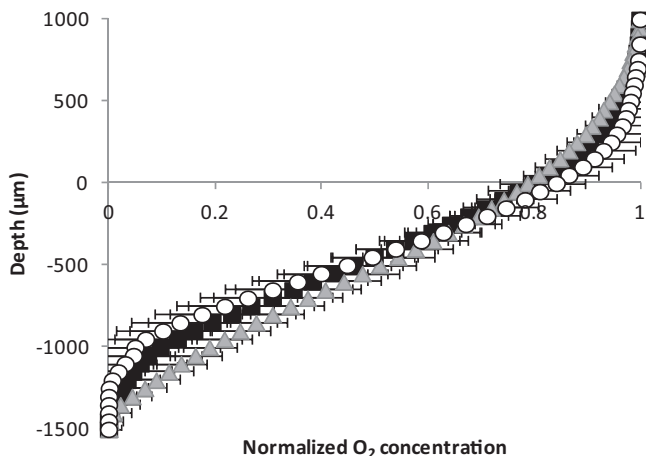


Fig. 1. O₂ profiles measured at three different locations in *P. chrysosporium* biofilms cultivated for 4 days in the absence of SeO₃²⁻ (■) and then treated with 1.67 mg Se L⁻¹ (10 mg Se L⁻¹) as SeO₃²⁻ for 3 (▲) and 24 h (○).

Table 1

Parameters calculated for *P. chrysosporium* biofilms cultivated in the flow-cell reactors for 4 days in the absence of SeO_3^{2-} (untreated) and then treated with $1.67 \text{ mg Se L}^{-1} \text{ h}^{-1}$ (10 mg Se L^{-1}) as SeO_3^{2-} for 3 and 24 h.*

Incubation	Effective O_2 diffusion coefficient in the biofilm, D_f ($\text{cm}^2 \text{ s}^{-1}$)	Relative diffusivity, f_D **	Porosity, θ **	Density, ρ (kg m^{-3})**	O_2 flux, J_w ($\text{mg cm}^{-2} \text{ s}^{-1}$)**
Untreated	$8.8 \times 10^{-6} \pm 9.9 \times 10^{-7}$	0.44 ± 0.05	0.76 ± 0.03	35.4 ± 7.2	$4.9 \times 10^{-7} \pm 6.5 \times 10^{-8}$
Treated for 3 h	$9.9 \times 10^{-6} \pm 1.4 \times 10^{-6}$	0.49 ± 0.07	0.79 ± 0.04	29.5 ± 7.8	$4.4 \times 10^{-7} \pm 9.0 \times 10^{-8}$
Treated for 24 h	$7.5 \times 10^{-6} \pm 7.3 \times 10^{-7}$	0.37 ± 0.04	0.72 ± 0.02	46.5 ± 7.2	$3.9 \times 10^{-7} \pm 7.8 \times 10^{-8}$

Note:

* Triplicates of the measured profiles at three different locations were used for calculations ($n = 9$).

** Parameters were calculated based on the estimated effective diffusion coefficients from the experimental data according to Eqs. (4), (5), (6) as well as (1) and (2).

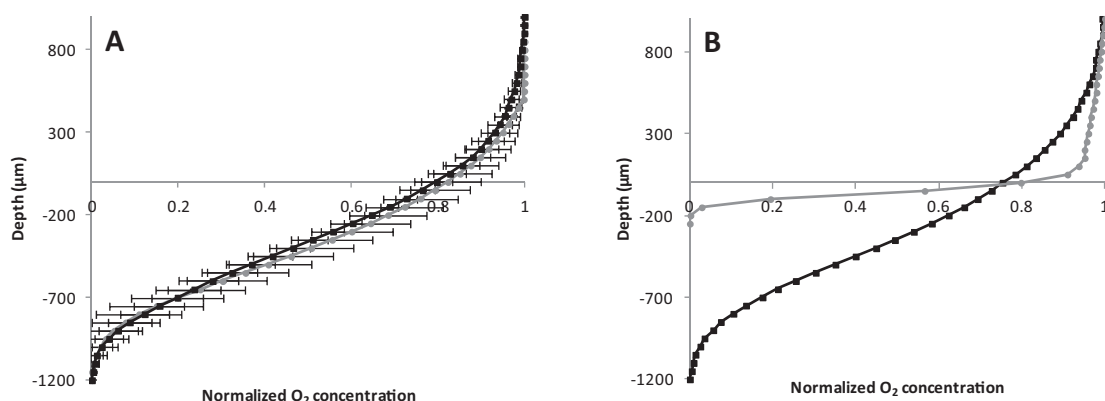


Fig. 2. Average oxygen profiles measured at: (A) different locations in biofilms grown in flow-cell reactors for 4 days in the absence (■) or presence of $1.67 \text{ mg Se L}^{-1} \text{ h}^{-1}$ (10 mg Se L^{-1}) as SeO_3^{2-} (●); (B) O_2 profiles measured in one location in biofilms grown in the flow-cell reactors for 4 days in the absence (■) or presence of $3.3 \text{ mg Se L}^{-1} \text{ h}^{-1}$ (20 mg Se L^{-1}) as SeO_3^{2-} (●).

was observed, which developed into overt differences in coloration intensity after 24 h. The change of biofilm coloration from white-ivory to orange-red is an indicator of the occurrence of SeO_3^{2-} reduction to red amorphous Se^0 (Espinosa-Ortiz et al., 2015; Sarkar et al., 2011). The intracellular synthesis of Se^0 from SeO_3^{2-} by *P. chrysosporium* was demonstrated in the fungal pellets (Espinosa-Ortiz et al., 2015), with formation of red, amorphous, spherical Se^0 particles in the nanometer range (30–400 nm). Although chemical characterization of the Se particles in the biofilm was not performed in the present study, the change of coloration upon SeO_3^{2-} exposure to red-orange in the *P. chrysosporium* biofilms strongly suggests that Se^0 formation occurred in the biofilms.

3.2. Influence of long-term SeO_3^{2-} exposure on *P. chrysosporium* biofilm activity and structure

3.2.1. Response to low Se influent concentration (10 mg Se L^{-1})

Fungal biofilms were grown for 4 days in the presence or absence of SeO_3^{2-} (10 mg L^{-1} , $1.67 \text{ mg Se L}^{-1} \text{ h}^{-1}$). O_2 concentration profiles were determined at three random locations in the biofilms. The O_2 concentration in the bulk solution varied from 7.0 to $7.5 \text{ mg O}_2 \text{ L}^{-1}$ (at 23°C). The profiles were normalized as described in Section 2.3. Average measurements for the untreated and Se-treated biofilms are shown in Fig. 2(A). Irrespective of the growth conditions, O_2 concentrations decreased to below the microsensor detection limit indicating that the biofilms developed anoxic zones. Table 2 provides the parameters obtained from the O_2 profile measurements in the Se-treated and untreated biofilms. These results suggest the effective O_2 diffusion coefficient being ~20% lower for the Se-treated biofilms. O_2 penetrated deeper into SeO_3^{2-} -treated biofilms, suggesting a decrease of the O_2 respiratory activity in the upper layers of the biofilms. This decrease was observed despite the estimated higher biofilm densities, which would other-

wise result in a smaller O_2 penetration depth due to higher O_2 consumption per unit volume in the biofilm in the absence of a lower respiratory activity. Based on Eq. (6), the biofilm density was estimated to be up to 30% greater compared to the untreated biofilms (Table 2), with an up to 7% decrease of the biofilm porosity. It should be noted that the density and porosity of the biofilms were not measured directly in this study, but rather estimated based on the diffusion coefficient estimates derived from the O_2 profiles (Fan et al., 1990; Zhang and Bishop, 1994). Thus, it cannot be stated conclusively whether density or porosity, or possibly both changed upon exposure to SeO_3^{2-} .

The global biofilm activity was also measured in terms of substrate consumption rate. The untreated biofilm showed a R_{glucose} value of $0.054 (\pm 0.005) \text{ g glucose L}^{-1} \text{ h}^{-1}$, corresponding to E_{glucose} of $52 (\pm 4.8)\%$, after 4 days of incubation. The Se-treated biofilm showed a R_{glucose} value of $0.044 (\pm 0.004) \text{ g glucose L}^{-1} \text{ h}^{-1}$ (E_{glucose} $41 \pm 4.3\%$). The difference in E_{glucose} between the untreated and Se-treated biofilms was statistically significant ($P_{\text{value}} = 0.041$), demonstrating that the incubation with SeO_3^{2-} decreases E_{glucose} in the biofilms. The removal of Se by the biofilm was estimated for the Se-treated biofilm, with an average R_{Se} of $0.32 (\pm 0.02) \text{ mg Se L}^{-1} \text{ h}^{-1}$ (E_{Se} $20 \pm 0.4\%$), after 4 days of incubation. At the end of the incubation period (4 days), the pH of the effluent decreased from 4.5 to $3.8 (\pm 0.02)$ for both Se-treated and untreated biofilms.

Upon 4 days of SeO_3^{2-} exposure, the biofilm showed an orange-red coloration, suggesting the biomineralization of Se^0 . A transversal cut (cross-section) of the biofilm showed an intensification of this orange-red coloration from the top to the bottom of the biofilm (data not shown). The upper zone of the biofilm showed either the white-ivory characteristic color of *P. chrysosporium* biofilms or a slight red coloration. Image analysis of the cross-section of the biofilm treated with SeO_3^{2-} (10 mg Se L^{-1} , $1.67 \text{ mg Se L}^{-1} \text{ h}^{-1}$) indicated a maximal thickness of $3500 \mu\text{m}$, providing an indication of the actual thickness of the biofilms grown in this study. The

Table 2
Parameters calculated for *P. chrysosporium* grown in the flow-cell reactors for 4 days in the absence (untreated) or presence of 1.67 mg Se L⁻¹ h⁻¹ (10 mg Se L⁻¹) as SeO₃²⁻.

Incubation	Effective O ₂ diffusion coefficient in the biofilm, D_f (cm ² s ⁻¹)	Relative diffusivity, f_D **	Porosity, θ **	Density, ρ (kg m ⁻³)**	O ₂ flux, J_w (mg cm ⁻² s ⁻¹)**
Untreated	$8.1 \times 10^{-6} \pm 4.8 \times 10^{-7}$	0.41 ± 0.02	0.74 ± 0.02	39.7 ± 4.5	$5.2 \times 10^{-7} \pm 3.0 \times 10^{-8}$
Treated	$6.6 \times 10^{-6} \pm 3.7 \times 10^{-7}$	0.32 ± 0.03	0.69 ± 0.03	56.6 ± 6.9	$4.2 \times 10^{-7} \pm 2.3 \times 10^{-8}$

Note:

* Triplicates of the measured profiles at three different locations were used for calculations ($n = 9$).

** Parameters were calculated based on the estimated effective diffusion coefficients from the experimental data according to Eqs. (4), (5), (6) as well as (1) and (2).

Table 3
Parameters calculated for biofilms of *P. chrysosporium* grown in the flow-cell reactors for 4 days in the absence (untreated) or presence of 3.3 mg Se L⁻¹ h⁻¹ (20 mg Se L⁻¹) as SeO₃²⁻.

Incubation	Effective O ₂ diffusion coefficient in the biofilm, D_f (cm ² s ⁻¹)	Relative diffusivity, f_D **	Porosity, θ **	Density, ρ (kg m ³)**	O ₂ flux, J_w (mg cm ⁻² s ⁻¹)**
Untreated	$8.9 \times 10^{-6} \pm 1.0 \times 10^{-7}$	0.44 ± 0.02	0.76 ± 0.02	35.3 ± 2.6	$5.2 \times 10^{-7} \pm 5.8 \times 10^{-8}$
Treated	$3.4 \times 10^{-6} \pm 2.0 \times 10^{-7}$	0.17 ± 0.03	0.55 ± 0.03	117.6 ± 21.5	$9.5 \times 10^{-7} \pm 5.4 \times 10^{-8}$

Note:

* Triplicates of the measured profiles at three different locations were used for calculations ($n = 9$).

** Parameters were calculated based on the estimated effective diffusion coefficients from the experimental data according to Eqs. (4–6) as well as (1) and (2).

heterogeneity and activity of the biofilm was clearly visible as a result of different intensities of the orange-red coloration, with particularly high color intensity in the region near the reactor's inlet, indicating gradients of Se⁰ accumulation and decreasing the amounts of Se⁰ with distance from the reactor inlet. Moreover, observations made on the cross-section of the 4 day-old Se-treated biofilm (10 mg Se L⁻¹) also indicated gradients of coloration with biofilm depth, with the most intense color at the bottom of the biofilm. This suggests that significant quantities of red amorphous Se⁰ accumulated in the deepest layers of the biofilm. Further studies to determine the distribution and speciation of Se species inside the biofilms are suggested, using for example grazing-angle X-ray spectroscopic techniques, X-ray standing wave (XSW) and X-ray absorption near edge structure (XANES) spectroscopy (Templeton et al., 2003), or X-ray fluorescence imaging (XFI) and scanning transmission X-ray microscopy (STXM) (Yang et al., 2016).

3.2.2. Response to high Se influent concentration (20 mg Se L⁻¹)

O₂ concentration profiles (Fig. 2B) were acquired after 4 days of incubation with twice the Se concentration used in the previous experiments (20 mg L⁻¹, 3.3 mg Se L⁻¹ h⁻¹). O₂ concentrations below the detection limit were observed in both the Se-treated and untreated biofilms (Fig. 2B). Table 3 summarizes the calculated parameters for the Se-treated and untreated biofilms based on the O₂ profiles. The estimate for the effective O₂ diffusion coefficient was 2.6 times lower for the Se-treated biofilm. Using Eq. (6), the biofilm density of the Se-treated biofilm was calculated to be 3.3 times higher than for the density of biofilms not exposed to SeO₃²⁻. Based on Eq. (5), the porosity of the Se-treated biofilms was estimated to decrease from 0.76 ± 0.02 to 0.55 ± 0.03 . For both the 10 and 20 mg Se L⁻¹ exposure (see Section 3.2.1), smaller diffusion coefficients were estimated compared to the untreated biofilms (Tables 2 and 3), suggesting high density structures and/or low biofilm porosities (Zhang and Bishop, 1994). The diffusive transport within biofilms generally decreases with increasing density and decreasing porosity of the biofilm (e.g. Fan et al., 1990).

Cryo-sectioning combined with microscopy indicated that *P. chrysosporium* biofilm formed in the presence of SeO₃²⁻ was considerably thinner (thickness $79 \pm 40 \mu\text{m}$) than the untreated biofilm (thickness $390 \pm 85 \mu\text{m}$) equivalent to a 80% reduction in biofilm thickness. It should be noted that the sample taken for the cryo-sectioning was randomly selected from the area where O₂ profiling was performed, but due to the biofilm heterogeneity, actual thickness could have varied. The difference between the biofilm thickness of the untreated biofilm in this experiment (390 μm)

and the estimate of 3500 μm for the long-term 10 mg Se L⁻¹ (1.64 mg Se L⁻¹ h⁻¹) exposure could be due to the differences in sample preparation, in which the biofilm might have contracted during sample preparation for microscopy (i.e. during freezing, harvesting or staining). The R_{glucose} values were $0.049 (\pm 0.004)$ and $0.034 (\pm 0.003) \text{ g L}^{-1} \text{ h}^{-1}$, corresponding to E_{glucose} values of $46 (\pm 4)$ and $32 (\pm 4)\%$ for the untreated and Se-treated biofilms, respectively. The differences between the R_{glucose} ($P_{\text{value}} = 0.006$) and E_{glucose} ($P_{\text{value}} = 0.012$) between the untreated and Se-treated biofilms were statistically significant, confirming the inhibition of substrate consumption in the biofilms due to SeO₃²⁻ exposure. The R_{Se} was $0.30 (\pm 0.17) \text{ mg Se L}^{-1} \text{ h}^{-1}$ ($E_{\text{Se}} = 6.6 \pm 2.5\%$).

The architecture of both untreated and Se-treated biofilms (SeO₃²⁻ exposure for 4 days, 20 mg L⁻¹, 3.3 mg Se L⁻¹ h⁻¹) was visualized using CLSM. The images of the untreated and Se-treated biofilms suggest that the structure of the biofilm was affected by the presence of SeO₃²⁻ (Fig. S2, Supplementary Material). While both untreated and Se-treated biofilms appeared to be composed of an elaborate network of hyphal elements, a more compact and dense structure was observed for the Se-treated samples. This confirms the densification of the Se-treated biofilms as suggested by the estimated overall biofilm density based on Eq. (6) and the decrease of the effective O₂ diffusion coefficient in the Se-treated biofilms (Table 3). Moreover, side views of the three-dimensional reconstructed CLSM images indicated denser and ~50% shorter hyphae in the Se-exposed biofilms (Fig. S2, Supplementary Material).

Overall, this study indicates that SeO₃²⁻ triggers the formation of a denser structure in *P. chrysosporium* biofilms. Densification and a possible decrease in the biofilm porosity of the Se-treated biofilms can be attributed to either a secondary response due to Se⁰ biomineralization or a toxic stress response to SeO₃²⁻ (mechanism of resistance). Microbially mediated mineralization might influence the local physical biofilm properties by increasing the volume fraction of minerals inside the biofilm and decreasing the local porosity.

Densification is a recognized mechanism of resistance in biofilms (Mah and O'Toole, 2001). Changes in biofilm structure, including the formation of more compact or dense biofilms, can be a biofilm stress response. The presence of metals induces the formation of stiffer or more compact bacterial biofilms. In a recent study, compaction was induced in *Xylella fastidiosa* when the biofilms were exposed to sub-inhibitory concentrations of Zn²⁺ (Navarrete and De la Fuente, 2014). In yeast biofilms of *Candida albicans* and *Candida tropicalis*, sub-inhibitory concentrations of metal ions (Zn²⁺, Co²⁺, Cu²⁺, Ag⁺, Cd²⁺, Hg²⁺) and metalloid oxyanions (AsO₄³⁻ and SeO₃²⁻) inhibited hyphal formation, leading to overt changes in biofilm structure (Harrison et al., 2007). The effect of

SeO₃²⁻ on the fungal morphology has already been demonstrated for *P. chrysosporium* pellets by Espinosa-Ortiz et al. (2015), wherein the authors demonstrated the occurrence of more compact, smaller and denser structures in the presence of Se. Stress due to toxicant exposure can increase the synthesis rate of extracellular polymeric substances (EPS) and/or change the nature and properties of EPS produced, which could lead to spatial restructuring of the biofilms. Dhanjal and Cameotra (2011) observed that SeO₃²⁻ exposure induces the production of large quantities of EPS in *Bacillus* sp., with different composition compared to cells cultivated in the absence of Se. Thus, the presence of SeO₃²⁻ could induce changes in the EPS production of *P. chrysosporium* biofilms and in turn modify the physical properties of the biofilms. Further research to assess the impact on EPS production and composition in fungal biofilms due to SeO₃²⁻ exposure is recommended as one of the next steps.

When biofilm densification occurs the microbial growth inside the biofilm appears to result in an increase of cell density rather than an increase in biofilm thickness (Ramsay et al., 1989). This could explain the noticeable decrease in biofilm thickness (~80%) when treated with 3.3 mg Se L⁻¹ h⁻¹ (20 mg Se L⁻¹) SeO₃²⁻. It should be noted that the cryo-sectioning method used in this study to estimate the thickness of the fungal biofilm (Villa et al., 2011) is most commonly used for thin biofilms (<500 µm) and might result in less accurate estimates for thicker biofilms (Yu et al., 1994 and discussed above). Image analysis of the transversal cut of the biofilm treated with 1.67 mg Se L⁻¹ h⁻¹ (10 mg Se L⁻¹) SeO₃²⁻, revealed a maximum thickness of 3500 µm, providing an indication of the actual thickness of the biofilms grown in this study. A decrease in biofilm thickness has been reported in biofilms as a response to the presence of toxicants or inhibitors. The thickness of *C. albicans* biofilms decreased by ~77% when incubated for 72 h in the presence of zosteric acid sodium salt, a well-known anti-biofilm compound (Villa et al., 2011).

4. Conclusions

The effects of SeO₃²⁻ on the O₂ respiratory activity and the physical properties of *P. chrysosporium* biofilms were evaluated using O₂ microensors combined with CLSM imaging. Short-term exposure to SeO₃²⁻ had an inhibitory effect on the respiratory activity of biofilms. The presence of SeO₃²⁻ showed a marked influence on the morphology of the biofilms, leading to the formation of a more compact and dense structures with, on average, shorter fungal hyphae, which is attributed to a mechanism of resistance or a secondary response due to Se⁰ biomineralization.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.biortech.2016.02.074>.

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