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

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28 **Abstract**

29 The temporal and spatial effects of selenite (SeO_3^{2-}) on the physical properties and
30 respiratory activity of *Phanerochaete chrysosporium* biofilms, grown in flow-cell reactors,
31 were investigated using oxygen microsensors and confocal laser scanning microscopy
32 (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
33 L^{-1} influent concentration), for 24 h, resulted in a 25% reduction of the O_2 flux, followed by
34 a ~10% decrease in the glucose consumption rate. Long-term exposure (4 days) to SeO_3^{2-}
35 influenced the architecture of the biofilm by creating a more compact and dense hyphal
36 arrangement resulting in a decrease of biofilm thickness compared to fungal biofilms grown
37 without SeO_3^{2-} . To the best of our knowledge, this is the first time that the effect of SeO_3^{2-}
38 on the aerobic respiratory activity on fungal biofilms is described.

39

40 **Key words:** Fungal biofilm, *Phanerochaete chrysosporium*, selenium, oxygen profiles,
41 microelectrodes

42 **1 Introduction**

43 Selenium (Se), an element belonging to group 16 of the periodic table (chalcogens),
44 plays a dual role as an essential micronutrient at low concentrations, but it can also be toxic
45 to organisms at higher concentrations (Lenz and Lens, 2009). An overdose of Se disrupts the
46 integrity of proteins and decreases cellular enzymatic activity, leading to severe
47 consequences for human health, including dermal, respiratory and neurological damage
48 (USHHS, 2003). Se is of significant research interest from an environmental viewpoint due
49 to its toxicity in natural water bodies. In particular, the water-soluble oxyanions selenite
50 (SeO_3^{2-}) and selenate (SeO_4^{2-}), mainly related to agricultural activities, mining, chemical,
51 textile, photographic and electronic industries, has shown to cause widespread impacts on
52 aquatic life, leading to a cascade of detrimental bioaccumulation (Chasteen and Bentley,
53 2003; Lemly, 2004; Lenz and Lens, 2009).

54 The use of biological agents to remove pollutants from water can be an efficient,
55 economic and environmentally friendly alternative to conventional physicochemical
56 methods, *e.g.* chemical precipitation, catalytic reduction and ion exchange. Different
57 microorganisms have the ability to convert SeO_3^{2-} and SeO_4^{2-} into insoluble forms, mainly
58 elemental Se (Se^0). The biomineralization of Se has been used for bioremediation of soils
59 and wastewater treatment, with the potential to recover Se biominerals (Nancharaiah and
60 Lens, 2015). Most of the reported biotechnologies for the treatment of Se polluted effluents
61 are based on bacterial metabolism (Nancharaiah and Lens, 2015), although the use of fungi
62 is also promising due to their ability to grow under acidic conditions, ease of handling and
63 their inherent capacity to produce high amounts of enzymes. The latter property is of
64 particular importance since the reduction of metal/metalloid ions and the formation of their
65 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 alteranata* (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 Materials and 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; $MgSO_4 \cdot 7H_2O$, 0.5; NH_4Cl , 0.1; $CaCl_2 \cdot 2H_2O$, 0.1; thiamine, 0.001; and 5 mL of a trace element solution as described by Tien and Kirk (1988). The initial

114 pH was adjusted to 4.5 with 1 M HCl, and the medium was sterilized at 123 kPa and at 121
115 °C for 30 min.

116 **2.2 Biofilm growth and exposure experiments**

117 Biofilms of *P. chrysosporium* were cultivated for 4 days in flow-cell reactors (15 cm
118 × 3.8 cm × 3.5 cm) run in parallel in the presence (Se-treated) or absence (untreated) of
119 SeO_3^{2-} (Figure S1, Supplementary material). Each reactor had a working volume of 0.12 L
120 and a substratum surface area of 57 cm². The reactors were filled with medium (2.5 g glucose
121 L⁻¹, pH 4.5, T 30 °C) and the fungus was allowed to grow in batch conditions for one day to
122 facilitate the attachment of the cells. The reactors were then supplied with influent at a
123 constant flow rate ($Q_i = 0.02 \text{ L h}^{-1}$) for 3 days to attain a nominal hydraulic retention time
124 (HRT_n) of 6 h. The effluent was recycled ($Q_r = 0.09 \text{ L h}^{-1}$, recirculation to influent ratio =
125 4.5:1) to provide well-mixed conditions in the reactor. Thus, an actual hydraulic retention
126 time (HRT_a) of 1.1 h was maintained. The substrate loading rate was maintained constant at
127 0.08 g glucose L⁻¹ h⁻¹, while the Se influent concentrations was either 10 or 20 mg Se L⁻¹,
128 corresponding to a loading rate of 1.67 or 3.3 mg Se L⁻¹ h⁻¹, respectively. The influent
129 concentrations of glucose and Se reported were measured in the feed medium prior to being
130 mixed in the inlet of the reactors with the recirculation flow (sampling port 1, see SI); these
131 values were used to estimate the removal rates and efficiencies in the reactor (see Section
132 2.5). Biofilms were cultivated in closed reactors using atmospheric air as headspace.
133 Whenever required, the reactors were opened for the measurement of O₂ profiles and care
134 was taken to minimize the potential of external contamination. Microscopic imaging
135 revealed no indication of bacterial or archaeal growth in the flow-cell reactors.

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

139 (1) *Short-term SeO_3^{2-} exposure tests.* Untreated 4 day-old biofilms were exposed to
140 SeO_3^{2-} (10 mg Se L^{-1} , 1.67 mg Se $\text{L}^{-1} \text{h}^{-1}$) for 3 h and 24 h. The O_2 profiles were determined
141 *in situ* before the biofilms were exposed to SeO_3^{2-} (untreated) and again after 3 h and 24 h
142 of SeO_3^{2-} exposure. The results from this experiment were used to determine the reduction
143 in O_2 consumption rate within the biofilm upon sudden SeO_3^{2-} exposure.

144 (2) *Long-term SeO_3^{2-} exposure tests.* Biofilms were cultivated for 4 days either in the
145 absence or presence of SeO_3^{2-} . The response of the biofilms to (a) low (10 mg Se L^{-1} , 1.67
146 mg Se $\text{L}^{-1} \text{h}^{-1}$), and (b) high (20 mg Se L^{-1} , 3.3 mg Se $\text{L}^{-1} \text{h}^{-1}$) influent concentrations was
147 determined. The SeO_3^{2-} concentration was doubled in experiment 2b to delineate the
148 influence of a different SeO_3^{2-} load on the biofilm. A physical characterization of these
149 biofilms was performed with CLSM. O_2 profiles were measured within the biofilms at the
150 end of the incubation period (4 d) of each experiment.

151 **2.3 Microsensor measurements**

152 Microsensors for O_2 measurements were used to determine the influence of SeO_3^{2-} on
153 O_2 concentrations within the *P. chrysosporium* biofilms. O_2 profiles over the depth of the
154 biofilm at three different locations in the bioreactor were measured using a Clark-type O_2
155 microsensor with a tip diameter of 8-12 μm (Unisense, Denmark), having a detection limit
156 of 0.01 mg $\text{O}_2 \text{L}^{-1}$. Measurements were made *in situ*, by maintaining the flow continuously
157 in the bioreactors, at room temperature and under atmospheric O_2 conditions. Positioning of
158 the microsensors was done using a micromanipulator fitted with a motor controller, and the
159 sensor output data was collected online using the SensorTrace Pro software (Unisense,

Denmark). O₂ profiles were acquired in triplicate at each location. The profiles obtained were normalized by dividing the O₂ concentration measured at depth *z* by the O₂ concentration in the bulk solution. The observation of similar O₂ profiles for each triplicate suggested that pseudo-steady-state O₂ uptake was reached. Based on the O₂ 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 O₂ in the biofilms were estimated from the O₂ profiles. Obeying Fick's law, and considering one-dimensional diffusion gradients in the biofilm, the O₂ flux in the boundary layer between the bulk solution and the biofilm was estimated according to Equation 1. The O₂ flux in the biofilm was determined using Equation 2 (Lewandowski et al., 1991):

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

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

where D_w (cm² s⁻¹) is the O₂ diffusion coefficient in water and D_f (cm² s⁻¹) is the diffusion coefficient within the biofilm. dC/dz is the gradient O₂ concentration measured with the microsenors 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 Equation 3 (Wanner et al., 2006):

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

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

$$D_f = f_D D_w \quad \text{Eq. (4)}$$

183 **2.3.2 Estimation of biofilm physical properties based on diffusion coefficients and O₂**
184 **inhibition**

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

189
$$\theta = \sqrt[3]{f_D}$$
 Eq. (5)

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

194
$$\frac{D_f}{D_w} = 1 - \frac{0.43 \rho^{0.92}}{11.19 + 0.27 \rho^{0.99}}$$
 Eq. (6)

195 The global inhibition, I_j (%), *i.e.* the reduction in O₂ flux into the biofilms, and thus O₂
196 consumption rate, in the presence of SeO₃²⁻ relative to the control biofilms, was estimated
197 according to Equation 7 (Zhou et al., 2011):

198
$$I_j = 1 - \left(\frac{J_{W\ Se-Treated}}{J_{W\ Untreated}} \right)$$
 Eq. (7)

199 **2.4 Biofilm sectioning and imaging**

200 Samples from biofilms grown for 4 days in the absence or presence of SeO₃²⁻ (20 mg
201 Se L⁻¹, 3.3 mg Se L⁻¹ h⁻¹) were chosen randomly and stained with the fluorescent probe FUN
202 1 (Molecular Probes), as previously described elsewhere (Villa et al., 2011). Briefly, the
203 biofilms were stained by covering the biofilm samples with 30 μ M FUN 1 in phosphate
204 buffered saline (PBS) solution at 30 °C for 30 min in the dark. The biofilms were then rinsed
205 with PBS solution. The stained samples were covered with optimum cutting temperature

206 formulation (OCT, Tissue-Tek, USA) and frozen with dry ice. The frozen core was
 207 embedded in more OCT, and then sectioned at -20 °C using a Leica CM1850 cryostat. Cryo-
 208 sections (7 µm thick) were visualized with a Nikon Eclipse E800 microscope in transmission
 209 mode using differential interference contrast optics. Images were processed using the
 210 software MetaMorph (Molecular Devices, Downingtown, PA). Biofilm architecture of the
 211 stained biofilms was visualized using CLSM (Leica TCS-SP2 AOBS). The software Imaris
 212 (Bitplane Scientific Software, Zurich, Switzerland) was used for processing the images.

213 **2.5 Analytical methods**

214 Influent and effluent samples were collected twice per day and centrifuged at 4700 g
 215 for 15 min, and the supernatant was used for analysis. Glucose concentrations were
 216 determined using the dinitrosalicylic acid method (Miller, 1959) with D-glucose as the
 217 standard. The total Se concentration was measured using ICP-MS (Agilent 7500ce) after
 218 preserving the samples with 5% HNO₃ (Espinosa-Ortiz et al., 2015).

219 **2.6 Performance parameters of the flow-cell reactor**

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

$$223 \quad \text{Removal rate, } R_i (\text{mg L}^{-1} \text{ h}^{-1}) = \frac{Q [C_0 - C_t]}{V} \quad \text{Eq. (8)}$$

$$224 \quad \text{Removal efficiency, } E_i (\%) = \frac{C_0 - C_t}{C_0} \quad \text{Eq. (9)}$$

225 where *Q* (L h⁻¹) is the influent flow rate, *C*₀ (mg L⁻¹) is the influent concentration, *C*_{*t*} (mg L⁻¹)
 226 is the effluent concentration measured at time *t*, and *V* (L) is the working volume of the
 227 reactor (0.120 L). The subscript *i* in the equations indicates either Se or glucose.

228 2.7 Statistical analysis

229 Substrate and Se removal rates by *P. chrysosporium* biofilms are reported in this study.
230 Averages and standard deviations were calculated for the long term experiments when steady
231 state conditions were attained over the 4 day period of incubation. The effect caused by the
232 presence of SeO_3^{2-} on the E_i was evaluated by performing analysis of variance (ANOVA) at
233 $P_{\text{value}} \leq 0.05$.

234 3 Results and Discussion

235 3.1 Influence of short-term SeO_3^{2-} exposure on *P. chrysosporium* biofilm activity

236 Short-term experiments were performed to ascertain the temporal effects of SeO_3^{2-} on
237 the *P. chrysosporium* biofilms, allowing to estimate the inhibition of the global O_2
238 consumption. Se-free biofilms were grown for 4 days and then exposed to SeO_3^{2-} (10 mg Se
239 L^{-1} , 1.67 mg Se $\text{L}^{-1} \text{h}^{-1}$), for 3 and 24 h. O_2 concentration profiles were determined at different
240 locations in the biofilm, before and after exposure to SeO_3^{2-} (Figure 1). The O_2 concentration
241 in the bulk solution varied between 7.0 and 7.5 mg $\text{O}_2 \text{L}^{-1}$. Triplicates of the O_2 profiles were
242 acquired; similar profiles were obtained during the measurements of each location (data not
243 shown), which suggested a pseudo-steady-state O_2 uptake by the biofilm.

244 Table 1 summarizes the parameters obtained from the O_2 profiles. Before and after
245 SeO_3^{2-} exposure, O_2 concentrations reached levels below the detection limit in the *P.*
246 *chrysosporium* biofilm indicating the presence of anoxic zones within the biofilm (Figure
247 1). Differences in the O_2 profiles observed over time revealed the influence of SeO_3^{2-} on the
248 O_2 flux into the biofilm. While the differences between I_j values observed were not
249 statistically different ($P_{\text{value}} = 0.13$), a decrease in O_2 consumption rate of 11.5 (± 6.5)% after
250 SeO_3^{2-} exposure for 3 h and 20 (± 5.3)% upon exposure for 24 h was observed. The rates of
251 glucose consumption (R_{glucose}) were estimated to be 0.052, 0.049 and 0.047 g glucose $\text{L}^{-1} \text{h}^{-1}$

252 after 0, 3 and 24 h of SeO_3^{2-} treatment, respectively, corresponding to a 6% and 10% decrease
253 in R_{glucose} . The rates of selenium removal (R_{Se}) were calculated to be $0.17 \text{ mg Se L}^{-1} \text{ h}^{-1}$ after
254 3 h and $0.22 \text{ mg Se L}^{-1} \text{ h}^{-1}$ after 24 h, corresponding to E_{Se} values of 12% and 15.5%,
255 respectively.

256 The inhibition of the O_2 respiration and the decrease in the R_{glucose} of the biofilm
257 indicate that SeO_3^{2-} exposure is inhibitory to the activity of *P. chrysosporium*. Even though
258 O_2 profiles varied across the length of the reactor, which was attributed to the heterogeneous
259 nature of the biofilm, a clear inhibition in O_2 consumption was observed in the presence of
260 SeO_3^{2-} . I_j did not vary significantly over time, suggesting that the inhibitory response of *P.*
261 *chrysosporium* occurs within 3 h of SeO_3^{2-} exposure. This agrees with observations by Zhou
262 et al. (2011) who observed a 29% inhibition of O_2 consumption within 1 h of exposure of
263 wastewater biofilms to Zn^{2+} (5 mg L^{-1}). Analogous responses were observed in aerobic
264 wastewater biofilms exposed to ZnO nanoparticles (Hou et al., 2014), wherein the O_2
265 respiration activity in the outer layer of the biofilm was inhibited (27%) within 2 h of ZnO
266 nanoparticles exposure (50 mg L^{-1}).

267 Walters et al. (2003) showed stronger inhibitory effects of antibiotics ($10 \text{ } \mu\text{g mL}^{-1}$ of
268 ciprofloxacin or tobramycin) on cells in the more oxygenated areas of *P. aeruginosa*
269 biofilms, whereas the bacteria in the anoxic zones were more resistant. This was attributed
270 to low metabolic activity in the anoxic basal layer of the biofilm. Further investigations
271 should be performed in order to determine the local metabolic activity of the *P.*
272 *chrysosporium* biofilm. This could for instance be achieved by visualizing spatial patterns
273 of protein synthetic activity within the biofilm using epifluorescence microscopy as
274 demonstrated by Walters et al. (2003).

275

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

284 The inhibitory effect of SeO₃²⁻ on fungal growth has been previously described for
285 unicellular, polymorphic and filamentous fungi (Gharieb et al., 1995; Espinosa-Ortiz et al.,
286 2015). The response of *P. chrysosporium* biofilms to SeO₃²⁻ exposure was similar to the
287 responses observed for *P. chrysosporium* pellets (Espinosa-Ortiz et al., 2015). When grown
288 as pellets in the presence of SeO₃²⁻ (10 mg Se L⁻¹, 10 g glucose L⁻¹, pH 4.5, 30 °C, 12 days
289 incubation), the biomass production of the fungus decreased by ~80% compared to Se-free
290 incubations, while the glucose consumption was limited to ~30%.

291 An orange-red coloration developed in the biofilm over time during SeO₃²⁻ exposure
292 (not shown). After 3 h, a visible coloration was observed, which developed into overt
293 differences in coloration intensity after 24 h. The change of biofilm coloration from white-
294 ivory to orange-red is an indicator of the occurrence of SeO₃²⁻ reduction to red amorphous
295 Se⁰ (Espinosa-Ortiz et al., 2015; Sarkar et al., 2011). The intracellular synthesis of Se⁰ from
296 SeO₃²⁻ by *P. chrysosporium* was demonstrated in the fungal pellets (Espinosa-Ortiz et al.,
297 2015), with formation of red, amorphous, spherical Se⁰ particles in the nanometer range (30-
298 400 nm). Although chemical characterization of the Se particles in the biofilm was not
299 performed in the present study, the change of coloration upon SeO₃²⁻ exposure to red-orange

300 in the *P. chrysosporium* biofilms strongly suggests that Se^0 formation occurred in the
301 biofilms.

302 **3.2 Influence of long-term SeO_3^{2-} exposure on *P. chrysosporium* biofilm activity and** 303 **structure**

304 **3.2.1 Response to low Se influent concentration (10 mg Se L^{-1})**

305 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
306 in the biofilms. The O_2 concentration in the bulk solution varied from 7.0 to $7.5 \text{ mg O}_2 \text{ L}^{-1}$
307 (at 23°C). The profiles were normalized as described in Section 2.3. Average measurements
308 for the untreated and Se-treated biofilms are shown in Figure 2A. Irrespective of the growth
309 conditions, O_2 concentrations decreased to below the microsensor detection limit indicating
310 that the biofilms developed anoxic zones. Table 2 provides the parameters obtained from the
311 O_2 profile measurements in the Se-treated and untreated biofilms. These results suggest the
312 effective O_2 diffusion coefficient being $\sim 20\%$ lower for the Se-treated biofilms. O_2
313 penetrated deeper into SeO_3^{2-} -treated biofilms, suggesting a decrease of the O_2 respiratory
314 activity in the upper layers of the biofilms. This decrease was observed despite the estimated
315 higher biofilm densities, which would otherwise result in a smaller O_2 penetration depth due
316 to higher O_2 consumption per unit volume in the biofilm in the absence of a lower respiratory
317 activity. Based on Equation 6, the biofilm density was estimated to be up to 30% greater
318 compared to the untreated biofilms (Table 2), with an up to 7% decrease of the biofilm
319 porosity. It should be noted that the density and porosity of the biofilms were not measured
320 directly in this study, but rather estimated based on the diffusion coefficient estimates
321 derived from the O_2 profiles (Fan et al., 1990; Zhang and Bishop, 1994). Thus, it cannot be
322

323 stated conclusively whether density or porosity, or possibly both changed upon exposure to
324 SeO_3^{2-} .

325 The global biofilm activity was also measured in terms of substrate consumption rate.
326 The untreated biofilm showed a R_{glucose} value of $0.054 (\pm 0.005) \text{ g glucose L}^{-1} \text{ h}^{-1}$,
327 corresponding to E_{glucose} of $52 (\pm 4.8)\%$, after 4 days of incubation. The Se-treated biofilm
328 showed a R_{glucose} value of $0.044 (\pm 0.004) \text{ g glucose L}^{-1} \text{ h}^{-1}$ ($E_{\text{glucose}} 41 \pm 4.3\%$). The difference
329 in E_{glucose} between the untreated and Se-treated biofilms was statistically significant ($P_{\text{value}} =$
330 0.041), demonstrating that the incubation with SeO_3^{2-} decreases E_{glucose} in the biofilms. The
331 removal of Se by the biofilm was estimated for the Se-treated biofilm, with an average R_{Se}
332 of $0.32 (\pm 0.02) \text{ mg Se L}^{-1} \text{ h}^{-1}$ ($E_{\text{Se}} 20 \pm 0.4\%$), after 4 days of incubation. At the end of the
333 incubation period (4 days), the pH of the effluent decreased from 4.5 to $3.8 (\pm 0.02)$ for both
334 Se-treated and untreated biofilms.

335 Upon 4 days of SeO_3^{2-} exposure, the biofilm showed an orange-red coloration,
336 suggesting the biomineralization of Se^0 . A transversal cut (cross-section) of the biofilm
337 showed an intensification of this orange-red coloration from the top to the bottom of the
338 biofilm (data not shown). The upper zone of the biofilm showed either the white-ivory
339 characteristic color of *P. chrysosporium* biofilms or a slight red coloration. Image analysis
340 of the cross-section of the biofilm treated with SeO_3^{2-} (10 mg Se L^{-1} , $1.67 \text{ mg Se L}^{-1} \text{ h}^{-1}$)
341 indicated a maximal thickness of $3500 \mu\text{m}$, providing an indication of the actual thickness
342 of the biofilms grown in this study. The heterogeneity and activity of the biofilm was clearly
343 visible as a result of different intensities of the orange-red coloration, with particularly high
344 color intensity in the region near the reactor's inlet, indicating gradients of Se^0 accumulation
345 and decreasing the amounts of Se^0 with distance from the reactor inlet. Moreover,
346 observations made on the cross-section of the 4 day-old Se-treated biofilm (10 mg Se L^{-1})
347 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 Se^0 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)*

O_2 concentration profiles (Figure 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). O_2 concentrations below the detection limit were observed in both the Se-treated and untreated biofilms (Figure 2B). Table 3 summarizes the calculated parameters for the Se-treated and untreated biofilms based on the O_2 profiles. The estimate for the effective O_2 diffusion coefficient was 2.6 times lower for the Se-treated biofilm. Using Equation 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 SeO_3^{2-} . Based on Equation 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 (Hibiya et al., 2004) 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 SeO_3^{2-} 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

372 thickness. It should be noted that the sample taken for the cryo-sectioning was randomly
373 selected from the area where O₂ profiling was performed, but due to the biofilm
374 heterogeneity, actual thickness could have varied. The difference between the biofilm
375 thickness of the untreated biofilm in this experiment (390 μm) and the estimate of 3500 μm
376 for the long-term 10 mg Se L⁻¹ (1.64 mg Se L⁻¹ h⁻¹) exposure could be due to the differences
377 in sample preparation, in which the biofilm might have contracted during sample preparation
378 for microscopy (*i.e.* during freezing, harvesting or staining). The $R_{glucose}$ values were 0.049
379 (± 0.004) and 0.034 (± 0.003) g L⁻¹ h⁻¹, corresponding to $E_{glucose}$ values of 46 (± 4) and 32 ($\pm$
380 4)% for the untreated and Se-treated biofilms, respectively. The differences between the
381 $R_{glucose}$ ($P_{value} = 0.006$) and $E_{glucose}$ ($P_{value} = 0.012$) between the untreated and Se-treated
382 biofilms were statistically significant, confirming the inhibition of substrate consumption in
383 the biofilms due to SeO₃²⁻ exposure. The R_{Se} was 0.30 (± 0.17) mg Se L⁻¹ h⁻¹ ($E_{Se} = 6.6 \pm$
384 2.5%).

385 The architecture of both untreated and Se-treated biofilms (SeO₃²⁻ exposure for 4 days,
386 20 mg L⁻¹, 3.3 mg Se L⁻¹ h⁻¹) was visualized using CLSM. The images of the untreated and
387 Se-treated biofilms suggest that the structure of the biofilm was affected by the presence of
388 SeO₃²⁻ (Figure S2, Supplementary material). While both untreated and Se-treated biofilms
389 appeared to be composed of an elaborate network of hyphal elements, a more compact and
390 dense structure was observed for the Se-treated samples. This confirms the densification of
391 the Se-treated biofilms as suggested by the estimated overall biofilm density based on
392 Equation 6 and the decrease of the effective O₂ diffusion coefficient in the Se-treated
393 biofilms (Table 3). Moreover, side views of the three-dimensional reconstructed CLSM
394 images indicated denser and ~50% shorter hyphae in the Se-exposed biofilms (Figure S2,
395 Supplementary material).

396 Overall, this study indicates that SeO_3^{2-} triggers the formation of a denser structure in
397 *P. chrysosporium* biofilms. Densification and a possible decrease in the biofilm porosity of
398 the Se-treated biofilms can be attributed to either a secondary response due to Se^0
399 biomineralization or a toxic stress response to SeO_3^{2-} (mechanism of resistance). Microbially
400 mediated mineralization might influence the local physical biofilm properties by increasing
401 the volume fraction of minerals inside the biofilm and decreasing the local porosity.

402 Densification is a recognized mechanism of resistance in biofilms (Mah and O'Toole,
403 2001). Changes in biofilm structure, including the formation of more compact or dense
404 biofilms, can be a biofilm stress response. The presence of metals induces the formation of
405 stiffer or more compact bacterial biofilms. In a recent study, compaction was induced in
406 *Xilena fastidiosa* when the biofilms were exposed to sub-inhibitory concentrations of Zn^{2+}
407 (Navarrete and De la Fuente, 2014). In yeast biofilms of *Candida albicans* and *C. tropicalis*,
408 sub-inhibitory concentrations of metal ions (Zn^{2+} , Co^{2+} , Cu^{2+} , Ag^+ , Cd^{2+} , Hg^{2+}) and metalloid
409 oxyanions (AsO_2^- and SeO_3^{2-}) inhibited hyphal formation, leading to overt changes in
410 biofilm structure (Harrison et al., 2007). The effect of SeO_3^{2-} on the fungal morphology has
411 already been demonstrated for *P. chrysosporium* pellets by Espinosa-Ortiz et al. (2015),
412 wherein the authors demonstrated the occurrence of more compact, smaller and denser
413 structures in the presence of Se. Stress due to toxicant exposure can increase the synthesis
414 rate of extracellular polymeric substances (EPS) and/or change the nature and properties of
415 EPS produced, which could lead to spatial restructuring of the biofilms. Dhanjal and
416 Cameotra (2011) observed that SeO_3^{2-} exposure induces the production of large quantities
417 of EPS in *Bacillus* sp., with different composition compared to cells cultivated in the absence
418 of Se. Thus, the presence of SeO_3^{2-} could induce changes in the EPS production of *P.*
419 *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 SeO_3^{2-} 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 $3.3 \text{ mg Se L}^{-1} \text{ h}^{-1}$ (20 mg Se L^{-1}) SeO_3^{2-} . 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 ($<500 \text{ }\mu\text{m}$) 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 $1.67 \text{ mg Se L}^{-1} \text{ h}^{-1}$ (10 mg Se L^{-1}) SeO_3^{2-} , revealed a maximum thickness of $3500 \text{ }\mu\text{m}$, 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 *Candida albicans* biofilms decreased by ~77% when incubated for 72 h in the presence of zoosteric acid sodium salt, a well-known anti-biofilm compound (Villa et al., 2011).

4 Conclusions

The effects of SeO_3^{2-} on the O_2 respiratory activity and the physical properties of *P. chrysosporium* biofilms were evaluated using O_2 microsensors combined with CLSM imaging. Short-term exposure to SeO_3^{2-} had an inhibitory effect on the respiratory activity of biofilms. The presence of SeO_3^{2-} 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 Se^0 biomineralization.

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451 **6 References**

- 452 1. Beheshti, N., Soflaei S., Shakibaie, M., Yazdi, M.H., Ghaffarifar, F., Dalimi, A.,
453 Shahverdi, A.R., 2013. Efficacy of biogenic selenium nanoparticles against *Leishmania*
454 major: in vitro and in vivo studies. *J Trace Elem. Med. Biol.* 27(3), 203–207.
- 455 2. Beyenal, H., Lewandowski, Z., 2002. Internal and external mass transfer in biofilms
456 grown at various flow velocities. *Biotechnol. Progr.* 18, 55–61.
- 457 3. Chasteen, T.G., Bentley, R., 2003. Biomethylation of selenium and tellurium:
458 microorganisms and plants. *Chem. Rev.* 103, 1–25.
- 459 4. Espinosa-Ortiz, E.J., Gonzalez-Gil, G., Saikaly, P.E., van Hullebusch, E.D., Lens, P.N.L.,
460 2015a. Effects of selenium oxyanions on the white-rot fungus. *Phanerochaete*
461 *chrysosporium*. *Appl. Microbiol. Biotechnol.* 99, 2405–2418.
- 462 5. Fan, L.S., Leyva-Ramos, R., Wisecarver, K.D., Zehner, B.J., 1990. Diffusion of phenol
463 through a biofilm grown on activated carbon particles in a draft-tube three-phase
464 fluidized-bed bioreactor. *Biotechnol. Bioeng.* 35, 279–286.
- 465 6. Flemming, H.C., Wingender, J., 2010. The biofilm matrix. *Nat. Rev. Microbiol.* 8, 623–
466 633.

- 467 7. Gharieb, M.M., Wilkinson, S.C., Gadd, G.M., 1995. Reduction of selenium oxyanions by
468 unicellular, polymorphic and filamentous fungi: Cellular location of reduced selenium
469 and implications for tolerance. *J. Ind. Microbiol.* 14, 300–311.
- 470 8. Harrison, J.J., Ceri, H., Stremick, C., Turner, R.J., 2004. Differences in biofilm and
471 planktonic cell mediated reduction of metalloid oxyanions. *FEMS Microbiol. Lett.* 235,
472 357–362.
- 473 9. Harrison, J.J., Ceri, H., Yerly, J., Rabiei, M., Hu, Y., Marinuzzi, R., Turner, R.J. 2007.
474 Metal ions may suppress or enhance cellular differentiation in *Candida albicans* and
475 *Candida tropicalis* biofilms. *Appl. Environ. Microbiol.* 73(1), 4940–4949.
- 476 10. Hou, J., Miao, L., Wang, C., Wang, P., Ao, Y., Qian, J., Dai, S., 2014. Inhibitory effects
477 of ZnO nanoparticles on aerobic wastewater biofilms from oxygen concentration profiles
478 determined by microelectrodes. *J. Hazard. Mater.* 276, 164–170.
- 479 11. Lauchnor, E.G., Semprini, L., 2013. Inhibition of phenol on the rates of ammonia
480 oxidation by *Nitrosomonas europaea* grown under batch, continuous fed, and biofilm
481 conditions. *Water Res.* 47, 4692–4700.
- 482 12. Lemly, A.D., 2004. Aquatic selenium pollution is a global environmental safety issue.
483 *Ecotoxicol. Environ. Safety* 59, 44–56.
- 484 13. Lenz, M., Lens, P.N.L., 2009. The essential toxin: The changing perception of selenium
485 in environmental sciences. *Sci. Total Environ.* 407, 3620–3633.
- 486 14. Lewandowski, Z., Walser, G., Characklis, W., 1991. Reaction kinetics in biofilms.
487 *Biotechnol. Bioeng.* 38, 877–882.
- 488 15. Mah, T.F.C., O'Toole, G.A., 2001. Mechanisms of biofilm resistance to antimicrobial
489 agents. *Trends Microbiol.* 9, 34–39.
- 490 16. Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing
491 sugar. *Anal. Chem.* 31, 426–428.

- 492 17. Morgenroth, E., 2008. Modelling biofilms. In: Henze M, van Loosdrecht MCM, Ekama
493 GA, Brdjanovic D. (eds) Biological wastewater treatment: principles, modelling and
494 design. IWA Publishing, London, UK, pp 457–497.
- 495 18. Nanchaiah, Y.V., Lens, P.N.L., 2015. Selenium biomineralization for biotechnological
496 applications. Trends Biotechnol. 33, 323–330.
- 497 19. Navarrete, F., De La Fuente, L. 2014. Response of *Xylella fastidiosa* to zinc: decreased
498 culturability, increased exopolysaccharide production, and formation of resilient biofilms
499 under flow conditions. Appl. Environ. Microbiol. 80:1097–1107.
- 500 20. Ramage, G., Rajendran, R., Sherry, L., Williams, C., 2012. Fungal biofilm resistance. Int.
501 J. Microbiol. 2012, 1–14.
- 502 21. Ramsay, J.A., Cooper, D.G., Neufeld, R.J., 1989. Effects of oil reservoir conditions on
503 the production of water-insoluble levan by *Bacillus licheniformis*. Geomicrobiol. 7, 155–
504 165.
- 505 22. Sarkar, J., Dey, P., Saha, S., Acharya, K., 2011. Mycosynthesis of selenium nanoparticles.
506 Micro. Nano. Lett. 6, 599–602.
- 507 23. Templeton, A.S., Trainor, T.P., Spormann, A.M., Brown, G.E., 2003. Selenium speciation
508 and partitioning within *Burkholderia cepacia* biofilms formed on α -Al₂O₃ surfaces.
509 Geochimica Cosmochimica Acta 67, 3547–3557.
- 510 24. USHHS. 2003. Toxicological profile for selenium. Toxicological Profile for Selenium;
511 2003. Available at: <http://www.atsdr.cdc.gov/toxprofiles/tp.asp?id=153&tid=28>
512 Accessed 10 November 2015.
- 513 25. van Hullebusch, E., Zandvoort, M.H., Lens, P.N.L., 2003. Metal immobilization by
514 biofilms: mechanisms and analytical tools. Rev. Environ. Sci. Biotech. 2, 9–33.
- 515 26. Vetchinkina, E., Loshchinina, E., Kursky, V., Nikitina, V., 2013. Reduction of organic
516 and inorganic selenium compounds by the edible medicinal basidiomycete *Lentinula*

517 *edodes* and the accumulation of elemental selenium nanoparticles in its mycelium. J.
518 Microbiol. 51, 829–835.

519 27. Villa, F., Pitts, B., Stewart, P.S., Giussani, B., Roncoroni, S., Albanese, D., Giordano, C.,
520 Tunesi, M., Cappitelli, F., 2011. Efficacy of zosteric acid sodium salt on the yeast biofilm
521 model *Candida albicans*. Microb. Ecol. 62, 584–598.

522 28. Walters III, M.C., Roe, F., Bugnicourt, A., Franklin, M.J., Stewart, P.S. 2003.
523 Contributions of antibiotic penetration, oxygen limitation, and metabolic activity to
524 tolerance of *Pseudomonas aeruginosa* biofilms to ciprofloxacin and tobramycin. Microb.
525 Agents Chemother. 47, 317–323.

526 29. Wanner, O., Eberl, H.J., Morgenroth, E., Noguera, D., Picioreanu, C., Rittmann, B.E., van
527 Loosdrecht, M., 2006. Mathematical modeling of biofilms. IWA Publishing, Hove, UK.

528 30. Wäsche, S., Horn, H., Hempel, D.C., 2002. Influence of growth conditions on biofilm
529 development and mass transfer at the bulk/biofilm interface. Water Res. 36, 4775–4784.

530 31. Yang, S.I., George, G.N., Lawrence, J.R., Kaminskyj, S.G.W., Dynes, J.J., Lai, B.,
531 Pickering, I.J., 2016. Multispecies biofilms transform selenium oxyanions into elemental
532 selenium particles: studies using combined synchrotron X-ray fluorescence imaging and
533 scanning transmission X-ray microscopy. Environ. Sci. Technol. Just accepted
534 manuscript. DOI: 10.1021/acs.est.5b04529

535 32. Yu, F.P., Callis, G.M., Stewart, P.S., Griebel, T., McFeters, G.A., 1994. Cryosectioning of
536 biofilms for microscopic examination. Biofiling 8, 85–91.

537 33. Zhou, X.H., Tong, Y., Han-Chang, S., Hui-Ming, S., 2011. Temporal and spatial
538 inhibitory effects of zinc and copper on wastewater biofilms from oxygen concentration
539 profiles determined by microelectrodes. Water Res. 45, 953–959.

540 34. Zhang, T.C., Bishop, P.L., 1994. Evaluation of tortuosity factors and effective
541 diffusivities in biofilms. Wat. Res. 28, 2279–2287.

542 35. Zhang, X., Yan, S., Tyagi, R.D., Surampalli, R.Y., 2011. Synthesis of nanoparticles by
543 microorganisms and their application in enhancing microbiological reaction rates.
544 Chemosphere 82: 489–494.
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List of figure captions

Figure 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 h⁻¹ (10 mg Se L⁻¹) as SeO₃²⁻ for 3(▲) and 24 h (○).

Figure 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 mg Se L⁻¹ h⁻¹ (10 mg Se L⁻¹) as SeO₃²⁻ (●); (B) O₂ profiles measured in one location in biofilms grown in the flow-cell reactors for 4 days in the absence (■) or presence of 3.3 mg Se L⁻¹ h⁻¹ (20 mg Se L⁻¹) as SeO₃²⁻ (●).

Figure 1.

Figure 2.

Tables

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, $\rho (\text{kg m}^{-3})^{**}$	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 Equations 4, 5, 6 as well as 1 and 2.

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 Equations 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	8.9×10 ⁻⁶ ±1.0×10 ⁻⁷	0.44±0.02	0.76±0.02	35.3±2.6	5.2×10 ⁻⁷ ±5.8×10 ⁻⁸
Treated	3.4×10 ⁻⁶ ±2.0×10 ⁻⁷	0.17±0.03	0.55±0.03	117.6±21.5	9.5×10 ⁻⁷ ±5.4×10 ⁻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 Equations 4, 5, 6 as well as 1 and 2.