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## **A compartmentalized microsystem helps understanding the uptake of benzo[a]pyrene by fungi during soil bioremediation processes**

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1 **Abstract**

2 Hydrophobic organic soil contaminants such as polycyclic aromatic hydrocarbons (PAH) are poorly  
3 mobile in the aqueous phase and tend to sorb to the soil matrix, resulting in low bioavailability. Some  
4 filamentous fungi are efficient in degrading this kind of pollutants. However, the mechanism of  
5 mobilization of hydrophobic compounds by non-motile microorganisms such as filamentous fungi  
6 needs investigations to improve pollutant bioavailability and bioremediation efficiency. Usual  
7 homogeneous media for microbial growth in the lab are poorly suited to model the soil, which is a  
8 compartmentalized and heterogeneous habitat.

9 A microfluidic device was designed to implement a compartmentalization of the fungal inoculum and  
10 the source of the pollutant benzo[a]pyrene (BaP) as a deposit of solid crystals in order to gain a  
11 further insight into the mechanisms involved in the access to the contaminant and its uptake in soils.  
12 Thus in this device, two chambers are connected by an array of parallel microchannels that are wide  
13 enough to allow individual hyphae to grow through them. Macro-cultures of *Talaromyces helicus* in  
14 direct contact with BaP have shown its uptake and intracellular storage in lipid bodies despite the low  
15 propensity of BaP to cross aqueous phases as shown by simulation. Observations of *T. helicus* in the  
16 microfluidic device through laser scanning confocal microscopy indicate preferential uptake of BaP at  
17 a close range and through contact with the cell wall. However faint staining of some hyphae before  
18 contact with the deposit also suggests an extracellular transport phenomenon. Macro-culture  
19 filtrates analyses have shown that *T. helicus* releases extracellular non-lipidic surface-active  
20 compounds able to lower the surface tension of culture filtrates to 49.4 mN/m. Thus, these results  
21 highlight the significance of active mechanisms to reach hydrophobic contaminants before their  
22 uptake by filamentous fungi in compartmentalized micro-environments and the potential to improve  
23 them through biostimulation approaches for soil mycoremediation.

24 **Key words**

25 Bioavailability; Biodegradation; Biosurfactant; Microfluidic device; Mycoremediation; Polycyclic  
26 aromatic hydrocarbons

## 27        **1 Introduction**

28    Soil is a complex environmental compartment resulting from the weathering of parental rock under  
29    the combined action of living organisms, surface water and the atmosphere. As a support for human  
30    activities, soil is heavily affected by pollution and notably with persistent pollutants, which is a major  
31    source of soil quality alteration (Panagos *et al.*, 2013). Among these pollutants, some families of  
32    organic contaminants including polycyclic aromatic hydrocarbons (PAH), polychlorobiphenyls, and  
33    various xenobiotics and pesticides, are highly hydrophobic and persistent in soils. Due to their  
34    carcinogenicity and high persistence in the environment, PAH are among the most regulated  
35    pollutants in environmental policies (Jennings, 2012). Such poorly water-soluble contaminants can be  
36    present in non-aqueous-phase liquids, tar or solid particles. Moreover, they tend to strongly sorb to  
37    soil aggregates and organic matter (Ortega-Calvo *et al.*, 2013). These properties make them poorly  
38    available for degradation by microorganisms (Posada-Baquero *et al.*, 2019).

39    Bioremediation corresponds to the use of biological activities in particular of microorganisms to  
40    destroy pollutants or decrease the associated risks for humans and the environment. It is regarded as  
41    a low-technology and low-cost approach with a higher degree of public acceptance in comparison to  
42    other physicochemical remediation techniques more destructive of the living fraction and the  
43    structure of soils such as high temperature incineration, chemical decomposition, solvent extraction  
44    or UV oxidation (Vidali, 2001). Thus bioremediation is one of the established methods undergoing the  
45    highest development for PAH cleanup in soils (Kuppusamy *et al.*, 2017). One of the parameters that  
46    can impair bioremediation efficiency is the low competitiveness and adaptability of microbial inocula  
47    (Rayu *et al.*, 2012), which could be prevented by the use of telluric strains. Soil is a divided  
48    environment consisting in solid, aqueous and gas phases, hosting a great variety of living organisms,  
49    including fungi. Among microorganisms, micromycetes are particular in the sense that they do not  
50    need water as a support for dispersal, and can form aerial structures. This allows mycelium to occupy  
51    vast volumes of soil and come into contact with several phases. Indeed, soil fungi are known for their  
52    ability to form mycelial networks in three dimensions that constitute large exchange surfaces within

53 the soil porosities, and to mobilize nutritious substrates through the release of extracellular lytic  
54 enzymes before substrates incorporation (Moore *et al.*, 2015). Notably, micromycetes play important  
55 roles in hydrophobic organic pollutants dynamics within the soil matrices (Baranger *et al.*, 2021).

56 Hydrocarbons can be a source of carbon and energy for fungi. In non-ligninolytic fungi, the main  
57 metabolic pathway described as involved in PAH biodegradation is the cytochrome P450 pathway.  
58 Cytochrome P450 mono-oxygenases are a family of intracellular enzymes involved in the oxidation of  
59 various hydrophobic substrates (Črešnar and Petrič, 2011). Cytochrome P450 monooxygenases  
60 initiate the oxidation of PAH into arene oxides that are further metabolized into phenol and  
61 dihydrodiol derivatives (Cerniglia and Sutherland, 2010). This intracellular degradation pathway  
62 implies preliminary uptake of hydrocarbons and notably PAH by fungal cells.

63 Lindley and Heydemann (1986) first described the incorporation of dodecanol into whole cells of  
64 *Cladosporium resinae* by early adsorption to the cell surface followed by active uptake. More recent  
65 studies have focused on the uptake of various hydrophobic organic pollutants including polycyclic  
66 aromatic hydrocarbons (PAH). PAH have been described to behave in a similar way to neutral lipids,  
67 diffusing into lipid membranes (Castelli *et al.*, 2002) and being incorporated in lipid storage sites in  
68 fungal cells (Verdin *et al.*, 2005; Chang *et al.*, 2015). Notably, the ubiquitous PAH benzo[a]pyrene (BaP)  
69 can be localized in lipid bodies in mycelium that has been cultivated in contact with this pollutant  
70 (Verdin *et al.*, 2005; Fayeulle *et al.* 2014). However, an active transport mechanism for BaP uptake  
71 dependent on the fungal cytoskeleton has been highlighted in *Fusarium solani*, and active  
72 phenomena may be involved in BaP mobilization before its uptake by the same fungus (Fayeulle *et al.*,  
73 2014). Interestingly, an active secondary transport mechanism was recently proposed for the uptake  
74 of monoaromatic compounds by *Phanerochaete chrysosporium* (Leriche-Grandchamp *et al.*, 2020).

75 Some micro-organisms are able to release biosurfactants, which can partially solubilize hydrophobic  
76 substrates and have been studied for the dispersal of hydrocarbons. In fungi, biosurfactants and  
77 emulsifiers are involved in nutrition for the mobilization of fats and adhesion to hydrophobic surfaces  
78 (Käppeli *et al.*, 1984). Modulations of cell surface hydrophobicity drive the attachment to surfaces

79 and utilization of hydrophobic substrates (plant leaf wax, oils...) and can affect the fungal interaction  
80 with hydrophobic pollutants (Puchkov *et al.*, 2002; Arutchelvi *et al.*, 2008; Garay *et al.*, 2018).  
81 Adsorption of hydrophobic substrates on the cell surface is thus thought to be a determining step  
82 prior to potential uptake by the cells (Fayeulle, 2013; Al-Hawash *et al.*, 2019). It is known that  
83 surfactants are released in the aqueous phase and accumulate at solid/water and air/water interfaces.  
84 However the interaction of fungal surfactants with hydrophobic soil contaminants and their potential  
85 contribution to pollutant mobilization is poorly understood.

86 *Talaromyces helicus* is a filamentous soil ascomycete with a cosmopolitan distribution, found on  
87 several continents in temperate to tropical climates (Huang and Schmitt, 1975; Romero *et al.*, 2009;  
88 Scervino *et al.*, 2010; Olagoke, 2014; Wu *et al.*, 2016; Fayeulle *et al.*, 2019). It can grow at moderate  
89 temperatures (20°C) and presents relatively fine hyphae of 1 to 3 µm in diameter (Baranger *et al.*,  
90 2020) able to penetrate microporosities and narrow cracks in the substrate, thus potentially covering  
91 large volumes of soil. This species has been isolated from contaminated soils, showing the resistance  
92 of some strains to pollutions, and its adaptation to the soil environment (Fayeulle *et al.*, 2019). Thus, *T.*  
93 *helicus* has been identified as holding potential for the bioremediation of multiple contaminations  
94 due to the bioaccumulation of heavy metals and the biodegradation of several organic pollutants  
95 including isoproturon, biphenyl and benzo[a]pyrene (Romero *et al.*, 2005, 2009, 2010; Fayeulle *et al.*,  
96 2019). The particular efficiency of *T. helicus* to improve PAH biodegradation in industrial soils with  
97 aged mixed contaminations was also highlighted at the microcosm scale (Fayeulle *et al.*, 2019).

98 In this study, we aim to investigate the biodegradation of BaP by *T. helicus* at the microscopic scale,  
99 which corresponds both to the size of fungal hyphae and to that of soil aggregates, and is therefore a  
100 natural scale to study soil microbial processes (Wilpiskeski *et al.*, 2019). Microfluidics offer the  
101 possibility to design model microenvironments with a good control of their geometry and chemical  
102 properties, taking advantage of the specific properties of small scale fluid flows to finely tune  
103 mechanical forces and mass transfers (Squires and Quake, 2005). Transparent microfluidic devices,  
104 usually made of glass or silicon rubber, can be used as soil models (Alekklett *et al.*, 2018) and

105 accommodate the growth of plant roots (Grossmann *et al.*, 2011), bacterial communities (Alnahhas *et*  
106 *al.*, 2019) or fungi (Held *et al.*, 2010). Parameters such as hyphal growth velocity can be measured by  
107 videomicroscopy and can be used to assess the ability of a fungal strain to colonize a polluted porous  
108 matrix (Baranger *et al.*, 2020).

109 In this work, we used a microfluidic device in which the model pollutant and the fungus were  
110 introduced in two separate compartments. The aim of such an experiment is to elucidate whether a  
111 filamentous fungus needs to grow in contact to the pollutant source for its uptake and degradation,  
112 or whether prior extracellular mobilization mechanisms are required to enhance the pollutant  
113 bioavailability in the aqueous phase. The investigation of this biological process is important for  
114 future optimizations of PAH mycoremediation protocols in soils through biostimulation approaches.  
115 This study is the first one to our knowledge to use a microfluidic device in order to study pollutants  
116 mobilization mechanisms by microorganisms in soil.

## 117 **2 Materials and methods**

### 118 **2.1 Fungal strain**

119 A strain of the filamentous fungus *Talaromyces helicus* from our laboratory collection and previously  
120 isolated from an industrial contaminated soil from North of France was used for this study.  
121 Identification of the strain occurred through molecular approach by BCCM™/MUCL (Louvain-la-Neuve,  
122 Belgium) based on sequencing ITS region, elongation factor gene, or  $\beta$ -tubulin gene, in complement  
123 of the macro- and micro-morphological features of pure cultures. The strain was maintained on MYEA  
124 solid medium (malt extract 20 g/L – Condalab, Madrid, Spain; yeast extract 2 g/L – VWR, Fontenay-  
125 sous-Bois, France; microbiological grade agar 15 g/L – Becton Dickinson, Rungis, France), at 22°C with  
126 a 12 h - 12 h light-dark cycle, and transplanted onto fresh medium every ten days.

127 Fresh mycelium was produced in a rich medium (MYPC) containing malt extract 10 g/L, yeast extract  
128 4 g/L, soy peptone 10 g/L (Merck, Darmstadt, Germany) and casamino-acids 2 g/L. Shaking flasks

129 containing 50 mL MYPC were inoculated with spores of *T. helicus* to reach a final concentration of  $10^4$   
130 spores/mL.

## 131 **2.2 Off-ship incubation with benzo[a]pyrene and lipid staining**

132 *T. helicus* cultures with BaP were prepared in mineral medium supplemented with glucose (MMG) at  
133 pH 5.5, as described by (Fayeulle *et al.*, 2019). MMG is comprised of KCl 0.25 g/L,  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$  1.54  
134 g/L,  $\text{Na}_2\text{HPO}_4$  8 mg/L,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.25 g/L,  $\text{NH}_4\text{NO}_3$  1 g/L,  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  1 mg/L,  $\text{MnCl}_2 \cdot \text{H}_2\text{O}$  0.1 mg/L,  
135  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  1 mg/,  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  0.5 mg/L,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  0.1 mg/L,  $\text{MoO}_3$  0.2 mg/L and glucose 20 g/L.  
136 Mineral salts and glucose were purchased from Thermo Fisher Scientific (Illkirch-Graffenstaden,  
137 France). Prior to adding the medium, 50  $\mu\text{g}$  of BaP (Sigma Aldrich, Haverhill, United Kingdom) was  
138 introduced in each flask by pipetting the correct amount of a BaP stock solution in acetone (0.8 g/L),  
139 and allowing the acetone to evaporate under a fume hood. Shaking flasks were inoculated with fresh  
140 mycelium of *T. helicus* that was pre-grown in MYPC, and were then incubated for 24h. After  
141 incubation, mycelium pellets were mounted on a microscope slide and observed in bright field and  
142 epifluorescence microscopy or in laser scanning confocal microscopy.

143 Lipids were stained *in vivo* on fresh mycelium samples taken from liquid cultures, using BODIPY  
144 500/510 lipid dye (Thermo Fischer Scientific – Illkirch-Graffenstaden, France). The stock solution of  
145 BODIPY in DMSO was pipetted directly onto mycelium samples mounted on a glass microscope slide,  
146 achieving a final dye concentration of 0.1  $\mu\text{M}$ /mL.

## 147 **2.3 Production and detection of surface-active compounds**

148 Shaking flasks containing MMG were inoculated with spores of *T. helicus* to reach a final  
149 concentration of  $10^4$  spores/mL. 50 mL flasks containing 10 mL medium were used for the growth  
150 kinetics experiment, and 250 mL flasks containing 50 mL medium were used for filtrates production  
151 before characterization. Cultures were incubated at 22°C with orbital shaking and a 12 h/12 h light-  
152 dark cycle. The mycelium was harvested by vacuum-filtration on quantitative filter paper (VWR 434)  
153 with a Büchner funnel. Filtrates were collected, filtered again on a 0.2  $\mu\text{m}$  syringe filter to sterilize



154 them and remove any remaining particle, and frozen at -20°C until use. To quantify fungal growth in  
155 10 mL cultures, the paper filters with mycelium cake were rinsed with distilled water and dried in a  
156 105°C oven for 24 h, then weighted. For the growth kinetics experiments, 10 mL cultures were done  
157 in triplicate and filtered separately.

158 Surface tension in filtrates was measured on a tensiometer (Krüss K-100) with the Wilhelmy plate  
159 method. Each measurement was taken 10 minutes after immersing the plate into the sample, to  
160 allow the meniscus to equilibrate at room temperature. All measurements were performed on  
161 triplicate samples, and results are presented as average values and standard deviations for each  
162 triplicate.

#### 163 **2.4 High-Performance Thin-Layer Chromatography**

164 High-Performance Thin-Layer Chromatography (HPTLC) analysis of culture filtrates was conducted by  
165 the CAP DELTA laboratory at Chromacim (Grabels, France) with the CAMAG method. Soy lecithin (1  
166 g/L), bovine serum albumin (1 g/L) and glucose (1 g/L) were used as positive controls for the  
167 detection of lipids, proteins and reducing sugars respectively. 10 µL of each control was deposited on  
168 the plate, as well as 50 µL of the crude filtrate. Samples were deposited on a TLC plate coated with  
169 F254 silica gel with an application spray ATS 4 and migrated vertically in a saturated ADC2 chamber  
170 over 20 min (migration distance of 70 mm). The mobile phase was a cosolvent mixture containing  
171  $\text{CHCl}_3:\text{CH}_3\text{OH}:\text{H}_2\text{O}:\text{NH}_3$  32 % (32:15:2:1 in volume).

172 After migration, thin layer plates were imaged under white light, UV 254 nm, UV 366 nm and scanned  
173 at 200 nm. Then a revelation was done with a primulin reagent (0.1 g/L in acetone:water 80:20 by  
174 volume) before imaging under white light, UV 366 nm and scanning at 366 nm for the detection of  
175 aliphatic carbon chains corresponding to lipids. Finally a revelation with an anisaldehyde reagent (4-  
176 methoxybenzaldehyde at 0.5% v/v in  $\text{CH}_3\text{OH}:\text{CH}_3\text{COOH}:\text{H}_2\text{SO}_4$  42.5:5:2.5 by volume) was carried out  
177 as well as imaging under white light and UV 366 nm for the detection of ketone, aldehyde and alcohol  
178 functions corresponding mostly to carbohydrates.

## 179        **2.5 Microfabrication**

180    Microfluidic chips for fungal culture were fabricated by soft lithography as described by Baranger *et al.*  
181    (2020). The pattern was imprinted on a silicon wafer using two layers of SU8 photoresist  
182    (Microfactory, IPGG, Paris): a first layer comprised of the microchannel pattern with an average  
183    thickness of  $5.8 \pm 0.3 \mu\text{m}$ , and a second layer forming the culture chambers, with an average  
184    thickness of  $124 \pm 2 \mu\text{m}$ . Polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning) mixed with 10%  
185    reticulating agent was cast against the wafer and allowed to cure for at least 2 h at 70°C. Inlets were  
186    added by punching holes through the PDMS with a biopsy puncher (2 mm for the inoculation well, 1  
187    mm for medium injection inlets). The negatively patterned PDMS slab was bound to a clean glass  
188    microscope slide or cover slip after surface oxidization in an oxygen plasma chamber (Harrick) for 60 s.

## 189        **2.6 On-chip fungal culture**

190    All chips were filled with sterile MMG prior to seeding with mycelium. A 2 mm diameter mycelium  
191    plug was collected from a solid-medium culture of *T. helicus* at the edge of the growing colony, and  
192    transferred to the inoculation inlet of the chip. The inlet was then closed with a PDMS plug to prevent  
193    drying. 1  $\mu\text{L}$  of BaP stock solution (0.8 g/L in acetone) was pipetted into one of the injection inlets on  
194    the opposite side to the inoculation chamber right after inoculation. Upon contact with the aqueous  
195    culture medium, BaP immediately precipitated into crystals localized at the injection inlet. The  
196    microchips were then placed in a sealed Petri dish in a water-saturated atmosphere. The mycelium  
197    was allowed to grow at 22°C with a 12 h - 12 h light-dark cycle in static conditions. Mycelial growth in  
198    the device and fluorescent staining of the cells due to BaP uptake were monitored in the chips for up  
199    to 8 days.

## 200        **2.7 Microscopy imaging and image processing**

201    Mycelia of *T. helicus* grown in liquid cultures with BaP and mounted between slide and coverslip were  
202    imaged in bright field and epifluorescence using an OLYMPUS BX60 microscope mounted with a color

203 Infinity 3-6UR camera (Lumenera). Time-lapse epifluorescence microscopy observations were  
204 performed using a DMI-8 inverted microscope (Leica) equipped with a motorized stage and a DFC  
205 3000G camera (Leica). The fluorescence of BaP was observed with a standard DAPI emission filter set.  
206 Microchips inoculated with *T. helicus* and spiked with BaP were imaged using a confocal laser  
207 scanning microscope (LSM 710 Axio Observer, Carl Zeiss). The fluorescent signal of BaP was detected  
208 using the DAPI filter (excitation 405 nm, emission 453 nm) and that of BODIPY using the FITC filter  
209 (excitation 488 nm, emission 563 nm).

210 All image processing and measurements were carried out using ImageJ. For the BODIPY co-staining  
211 experiment, mycelium mounted on a glass slide was imaged in confocal microscopy at 40x  
212 magnification with z increments of 0.38  $\mu\text{m}$ , and maximum intensity projections along z were made.  
213 For the on-chip fungal culture experiments, stacks of 11 images with a 1  $\mu\text{m}$  increment were  
214 projected along z. Average grey intensity was measured over rectangular areas of 10x100  $\mu\text{m}$  (12x121  
215 pixels) in 3 separate microchannels on each image. Results are presented as mean and standard  
216 deviation of the 3 measurements for each time point.

## 217 **2.8 In silico simulation**

218 The penetration of BaP through a model lipid bilayer was simulated using the IMPALA method as first  
219 described by Ducarme *et al.* (1998). Briefly, this method is based on a Monte Carlo approach using an  
220 implicit description of membrane. The latter is described as a continuous medium whose properties  
221 vary along the axis perpendicular to the bilayer plane (z axis). The force field was parameterized to  
222 mimic a membrane in aqueous environment by considering (1) the hydrophobic effect between the  
223 membrane and a solute (Epho) and (2) the perturbation effect of the solute on the lipid acyl chain  
224 organization (Elip). The two restraints were calculated and summed at each position of BaP into the  
225 implicit membrane and the molecule was systematically moved along the z axis by 1 Å steps, from  
226 one side of the membrane to the other. The method yields a profile of the interaction energy for  
227 each position along the Z axis, the most stable positions being those with minimal energy.

## 228        **3 Results**

### 229        **3.1. Benzo[a]pyrene diffusion in lipid phases**

230 BaP is highly hydrophobic ( $\log K_{ow} = 6.35$ ) and has an extremely low solubility in water ( $3.8 \cdot 10^{-3}$  mg/L  
231 at 25°C) (IARC, 2012). This compound forms solid crystals when added to aqueous media such as  
232 those used for microbial cultures. In soil, it is associated to solid particles and organic matter, but can  
233 also be present in hydrocarbon mixtures as non-aqueous phase liquids (Ortega-Calvo *et al.*, 2013).

234 The intracellular uptake of BaP in fungal cells implies that the molecule reaches cell surfaces and  
235 transport occurs through the plasma membrane. In order to predict the interaction of BaP with  
236 biological membranes, its penetration into an implicit symmetrical lipid bilayer was simulated by the  
237 IMPALA method (Fig. 2 a). As expected for a hydrophobic molecule, the profile displayed a sharp  
238 contrast between the positions corresponding to the water phase surrounding the membrane and  
239 those corresponding to the hydrocarbon core of the membrane. The most stable positions for BaP,  
240 displaying negative interaction energies, are located between -14 Å and 14 Å, and correspond to the  
241 alkyl core of the model lipid bilayer. In contrast, the interaction energy is maximal above -22 Å and  
242 22 Å, and decreases sharply between (-)22 Å and (-)14 Å. This decrease coincides with positions  
243 where the BaP molecule is in contact with the membrane, and still partially surrounded by water.  
244 Indeed, for a 36 Å thick membrane, the hydrated phosphate heads of the amphiphilic lipids are  
245 located between 14 and 18 Å.

246 The energy profile obtained predicts that the BaP molecule is most stable at the core of lipid bilayers,  
247 This result is consistent with the low solubility in water of BaP, which tends to minimize its contact  
248 surface with aqueous phases.

### 249        **3.2. Intracellular localization of benzo[a]pyrene in *Talaromyces helicus***

250 Since BaP emits a blue fluorescent signal when excited with UV light, its incorporation into the fungal  
251 biomass can be visualized directly through epifluorescence microscopy. Off-chip observations of *T.*  
252 *helicus* mycelium pellets incubated with BaP for 24 h were thus carried out. In control flasks without

253 BaP, a slight fluorescence of the mycelium was visible under UV (Fig. 2 b). The fluorescent signal  
254 observed without addition of any fluorophore corresponds to the autofluorescence of parietal  
255 components. Indeed, fungal hyphae are known to emit blue autofluorescence when exposed to UV  
256 light (330-385 nm) due to the presence of chitin in the fungal wall (Jabaji-Hare *et al.*, 1984; Dreyer *et*  
257 *al.*, 2006).

258 After 24 h of incubation with BaP, the mycelium pellets appeared brightly fluorescent at their core,  
259 which could be due to the immediate contact with BaP crystals trapped in the hyphal network. Blue  
260 fluorescence was detected along hyphae, with cell walls and septa appearing more strongly stained  
261 than the cytoplasm. Many intracellular vesicles were visible in bright field, and appeared brightly  
262 fluorescent. In some hyphal tips, these vesicles were distributed in the cytoplasm. In mature hyphae,  
263 the vesicles were localized at the periphery of the cytoplasm, while vacuoles were not stained (Fig. 2  
264 b). When mycelium previously incubated with BaP was stained with BODIPY and observed in confocal  
265 microscopy, blue and green fluorescent signals co-localized in lipid bodies indicating BaP intracellular  
266 storage in these organelles (Fig. 2 c).

### 267 **3.3. Detection of fungal surface-active molecules**

268 Culture filtrates of *T. helicus* were harvested and analyzed through surface tension measurements to  
269 detect potential fungal surfactants (Fig. 3 a). Surface tension in culture filtrates decreased over time  
270 and reached a minimum after 6 days, stabilizing until the end of the experiment (after 13 days). The  
271 surface tension was lowered to  $49.4 \pm 0$  mN/m, compared to  $69 \pm 3.7$  mN/m in fresh, sterile mineral  
272 medium. This result clearly demonstrates the presence of surface-active compounds in the  
273 extracellular medium. Extracellular proteins or biosurfactants secreted by the fungus may be  
274 responsible for this decrease.

275 In order to gather more information on the chemical nature of these surfactants, culture filtrates  
276 harvested after 13 days of incubation were analyzed in high performance thin-layer chromatography  
277 (Fig. 3.b). 13-day filtrates were chosen because they contain the lowest glucose contents, which can

278 interfere with the results and make it difficult to concentrate the samples. After migration, six main  
279 spots were detected through 254nm UV illumination, which reveals organic compounds in a non-  
280 specific way. The most intense spot was detected at 0.05 Rf and corresponds to residual glucose in  
281 the sample. Five fainter spots were detected at 0.08, 0.16, 0.38, 0.60 and 0.77 Rf. After spraying with  
282 a primulin solution to detect lipids and imaging at 266 nm, the only visible spot corresponded to the  
283 migration coefficient of glucose. Finally, anisaldehyde revelation was used to reveal carbohydrates.  
284 Spots at 0.08, 0.16, 0.60 Rf and the glucose spot reacted with anisaldehyde, but not the spots  
285 appearing at 0.60 and 0.77 Rf. In addition, a new spot at 0.72 Rf was visible in white light with  
286 anisaldehyde revelation. This compound may be present in concentrations too low for the spot to be  
287 visible without prior staining.

288 The filtrate is a mixture of several compounds with various affinities with the organic phase, which  
289 appears to be predominantly made of carbohydrates, and not lipids. Peptides were not investigated  
290 in HPTLC, however protein quantification in the filtrates through the Bradford method show the  
291 presence of extracellular protein in low amounts with the highest concentration at 3 mg/L after 10  
292 days of incubation.

### 293 **3.4 Benzo[a]pyrene mobilization in a compartmentalized device**

294 *T. helicus* was grown in microfluidic chips spiked with BaP, and the mycelium was observed at  
295 different growth stages. The mycelium was imaged in bright field to monitor growth and locate the  
296 edge of the colony. Chips were then observed in laser scanning confocal microscopy to detect the  
297 fluorescent staining of hyphae due to BaP incorporation.

298 Fig. 4 a shows the evolution of the fluorescent staining over time at two observations points in the  
299 same chip: in chamber A close to the inoculum (left column) and in chamber B right at the opening of  
300 microchannels (right column). Fig. 4 b represents the position of the different elements within the  
301 chip geometry. The intensity of the fluorescent staining increased over time. After 4 days of  
302 incubation, the mycelium had grown into the inoculation chamber A but did not yet reach chamber B.

303 Faint and diffuse staining of the hyphae was observed, but not more intense than the natural  
304 autofluorescence of the mycelium observed in control chips without BaP. After 5 days, hyphae  
305 reached the channels but not BaP crystals yet and the mycelium fluorescence appears still low. After  
306 6 days and even more after 7 days, staining intensity clearly increased and was not uniform across the  
307 mycelium, i.e. single stained hyphae were distinguishable and appeared more brightly fluorescent  
308 than others in contrast to the control (Fig. 4 a). No distinct stained vesicles were visible at 20x  
309 magnification. Microchannels enabled the quantification of grey intensity over time in the same area  
310 (Fig 4 c). The average fluorescence intensity appears to increase between 3 and 5 days, whereas it  
311 remains stable in the control over the whole experiment, which evokes a BaP uptake before direct  
312 contact between hyphae and BaP crystals occurred. However the difference of grey intensities  
313 between test with BaP and control starts to be statistically significant after 7 days when direct contact  
314 between the mycelium and the BaP deposit occurs.

315 In order to better locate the fluorescence within hyphae, several chips were observed at greater  
316 magnification (40X) after 6 and 7 days. Growth velocity was greatly variable from one chip to the  
317 other, as previously assessed in the same device with this fungal strain (Baranger *et al.*, 2020). As a  
318 result, after 7 days of incubation the mycelium had not reached the BaP deposit in all of the chips (Fig.  
319 5 a and b). In some of the chips where direct contact happened, staining of the mycelium was  
320 detected in chamber B. Stained apices were observed at the growth front, in close vicinity to the  
321 deposit (Fig. 5 c). In these apices, no lipid bodies were distinguishable and the whole cytoplasm  
322 appeared fluorescent. This could be due to the presence of numerous, closely packed lipid droplets  
323 that could not be resolved with the camera used. Indeed, the resolution of the images was 0.21  
324  $\mu\text{m}/\text{pixel}$  at 40x magnification, while some of the lipid droplets detected in stained apices with the  
325 Lumenera camera (used for epifluorescence images) could be as small as 0.2  $\mu\text{m}$ . In mature, ramified  
326 hyphae located in the chamber, and directly in contact with the crystals, stained vesicles were clearly  
327 visible (Fig. 5 d). Some hyphae in the microchannels were stained as well (not shown). The stained

328 structures were similar to those observed in mycelium grown in liquid medium supplemented with  
329 BaP, as described in paragraph 3.2.

330 Interestingly, in chips imaged before the mycelium had reached the deposit, fluorescent staining  
331 different from mycelia grown without BaP was observed as well. Indeed, faintly stained apices were  
332 visible at the growth front of the mycelium growing through the microchannels (Fig. 5 a). Some  
333 hyphae localized in chamber B, closer to the BaP deposit, displayed a stronger staining and visible  
334 intracellular lipid bodies (Fig. 5 b). The cell wall and some septa appeared to be stained as well. The  
335 stained lipid bodies were smaller and less numerous than they were in hyphae in contact with BaP  
336 crystals (Fig. 5 d).

## 337 **4 Discussion**

### 338 **4.1. Benzo[a]pyrene storage in lipid bodies**

339 Epifluorescence observations with a co-staining with BODIPY confirm that BaP is absorbed into the  
340 cells of *T. helicus* and stored in lipid bodies. Similar observations were made in the saprotrophic  
341 fungus *Fusarium solani* (Verdin *et al.*, 2005; Fayeulle *et al.*, 2014) as well as other fungal strains  
342 including the yeast *Saccharomyces cerevisiae*, the white rot *Phanerochaete chrysosporium* (Verdin *et*  
343 *al.*, 2005), and the oomycete *Pythium ultimum* (Furuno *et al.*, 2012). Intracellular PAH uptake thus  
344 appears to occur in several fungal species regardless of PAH degradation efficiency.

345 Accumulation of lipophilic toxins in lipid bodies may be a defense mechanism against oxidative stress  
346 in fungi. Indeed, Chang *et al.* (2015) found that the formation of large lipid bodies in *Candida albicans*  
347 was associated with mycotoxin resistance, and that toxins were stored in lipid bodies. The authors  
348 propose a protection mechanism against oxidative stress caused by the oxidation of aromatic rings,  
349 involving quenching of reactive oxygen species by the triacylglycerols making up the bulk of storage  
350 lipids. A similar strategy in *Talaromyces helicus* may serve as a protection against the adverse effects  
351 of BaP and other lipophilic organic compounds.



352 Additionally, lipid bodies may be a site of intracellular degradation. Delsarte *et al.* (2018) proposed a  
353 connection between BaP degradation and neutral lipid cycling in fungal cells, hypothesizing that BaP  
354 stored in lipid bodies could be oxidized simultaneously as lipid beta-oxidation occurs in neighboring  
355 peroxisomes.

#### 356 **4.2. Preferential uptake at a close range**

357 Previous hypotheses on the uptake mechanisms of hydrophobic compounds suggest that growing  
358 hyphal tips, which are metabolically active and host intense exchanges with the extracellular  
359 environment, are preferential sites of uptake (Fayeulle *et al.*, 2014). In accordance with this  
360 hypothesis, mycelium incubated with BaP frequently displays stained hyphal tips. Transport and  
361 storage in older parts of the mycelium is suspected but not established. Indeed, stained lipid bodies  
362 are visible in all parts of the mycelium, indicating that BaP is either incorporated in already formed  
363 segments, or brought there from the tips through intrahyphal transport. Intrahyphal translocation of  
364 PAH through vesicle streaming was observed in *P. ultimum* (Furuno *et al.*, 2012). However, although  
365 cytoplasmic streaming was observed during our time-lapse experiments in bright field microscopy, no  
366 evidence of lipid body movement was found in *T. helicus* with specific staining. Previous studies  
367 suggest that lipid bodies in filamentous fungi originate from the endoplasmic reticulum, forming  
368 small lipid bodies that later fuse into larger ones (Kamisaka *et al.*, 1999). Such early precursors of the  
369 mature lipid bodies may be more mobile, but smaller than the camera resolution used for the present  
370 experiments. Alternatively, BaP could be solubilized in membranes and be transported along with  
371 vesicles to various organelles, in a similar way to neutral lipids and other lipophilic compounds  
372 (Murphy *et al.*, 2009). Indeed, the IMPALA simulation results predict that the most stable position for  
373 BaP in a lipid bilayer is within the hydrophobic core, which indicate a possible penetration into cell  
374 membranes and subsequent lateral diffusion. The insertion of PAH into model lipid membranes was  
375 also demonstrated experimentally (Castelli *et al.*, 2002). However it is very unlikely for BaP in the  
376 molecular form to be present in water surrounding cells and to diffuse freely through the  
377 extracellular aqueous medium at concentrations high enough to explain its effective biodegradation.

378 Thus for BaP to be absorbed into membranes, it would need to be already in close vicinity to the cell  
379 surface, either as crystals, or solubilized by surface-active molecules. Transport phenomena, other  
380 than passive diffusion, must therefore occur earlier than the cell membrane crossing in the  
381 incorporation process to bring the pollutant in contact with the cell surface.

382 Microscopic observations of *T. helicus* in spiked microfluidic chips indicate a preferential uptake of  
383 BaP at a close range and through contact with the cell wall. Indeed, when BaP is supplied as a fixed  
384 solid deposit in microfluidic chambers, fluorescent staining of the mycelium is the most intense after  
385 the deposit has been reached. This is consistent with observations in mycelium suspensions as well,  
386 since, when BaP crystals are dispersed in the liquid medium through continuous agitation, they can  
387 come into direct contact with hyphae and get trapped into the tightly knit mycelial network. As a  
388 result, the core of mycelial pellets frequently appears brightly stained as opposed to growing hyphae  
389 at the periphery. Moreover, early staining of some hyphae was observed before contact with BaP  
390 crystals in microfluidic chips. The staining was most often localized in some hyphal apices, sometimes  
391 highlighting the cell wall of older hyphae and lipid bodies in the cytoplasm. These results indicate that  
392 a long-range mobilization mechanism is at play, bringing BaP molecules to the fungal cells before  
393 hyphae could reach the solid crystals. The staining of cell walls observed on some occasions suggests  
394 that BaP adsorption to cell walls is a preliminary step to further internalization. Accumulation of BaP  
395 at the cell wall was previously shown in *F. solani* (Fayeulle *et al.*, 2014) when the formation of actin  
396 filaments is inhibited, preventing endocytosis and vesicle trafficking. In a context of non-inhibited  
397 metabolic activity, little to no staining of the cell wall is observed, indicating an equilibrium between  
398 cell wall adsorption rates and uptake rates.

### 399 **4.3 Extracellular mobilization and transport**

400 The access to the BaP deposits in crystal form relies either on hyphal growth up to the source, or on  
401 transport in water. Surface-active compounds may increase BaP partitioning into the water phase by  
402 accumulating at the surface of solid BaP particles and promote their fragmentation and dispersion in

403 the medium. This hypothesis seems in accordance with the observation that surfactants enable  
404 transfers of hydrophobic molecules between hydrophobic phases of an emulsion stabilized by solid  
405 particles (Drelich *et al.*, 2012). However, the possible role of fungal surfactants in BaP mobilization  
406 has not been elucidated so far.

407 The mechanisms involved in hydrophobic pollutant mobilization could be closely linked to the  
408 nutrition behaviour of filamentous fungi. Indeed, hydrophobic compounds in contaminated soils such  
409 as BaP are frequently associated with non-aqueous phase liquids and organic matter, which can  
410 readily be used by fungi as carbon sources. Some microbial surfactants are able to partially solubilize  
411 hydrophobic organic compounds (Ron and Rosenberg, 2002; Van Hamme *et al.*, 2006), including PAH  
412 (Garcia-Junco *et al.*, 2003; Sánchez-Vázquez *et al.*, 2018), thus enhancing their bioavailability.  
413 Sophorolipid biosurfactants excretion has also been linked to the degradation of anthracene sorbed  
414 to a solid matrix in several yeast species (Romero *et al.*, 2016). However in our study with  
415 *Talaromyces helicus*, no extracellular lipids were detected, excluding sophorolipids or other similar  
416 glycolipids from biosurfactants candidates. Interestingly, non-lipidic surface-active molecules have  
417 been described to play major roles in fungal interactions with hydrophobic substrates such as  
418 hydrophobins (Berger and Sallada, 2019) or exopolysaccharides (Mahapatra and Banerjee, 2013). This  
419 kind of molecules could be related to the surface tension decrease of culture filtrates observed in our  
420 study. Dissolved organic matter itself can serve as a mobilizing agent promoting hydrophobic organic  
421 compounds (HOC) dispersion in the water phase (Smith *et al.*, 2009, 2011). Biosurfactants could also  
422 promote the dispersal and transport of small solid particles or droplets of organic phases containing  
423 BaP. Additionally, fungal hyphae whose growth was oriented towards the source of nutrients  
424 following a gradient, may thus come into contact with associated pollutants. Facilitated transport of  
425 HOC in small amounts in the aqueous phase would thus be a preliminary step to direct contact and  
426 incorporation in greater amounts.

427 Although the main PAH biodegradation pathway in non-ligninolytic fungi is thought to be intracellular,  
428 fungi release a variety of extracellular enzymes involved in nutrition, including oxidoreductases such

429 as laccases, lignin peroxidases, manganese peroxidases, and tyrosinases which catalyze the  
430 production of oxidizing agents and free radicals leading to the non-specific oxidation of substrates  
431 (Tuomela and Hatakka, 2011). The fungal metabolism of aromatic compounds produces substituted  
432 derivatives that are more reactive and more soluble than the original pollutants like phenol,  
433 phthalate, hydroxyl-, carboxy- and dihydrodiol derivatives that may in a second step be conjugated with  
434 sugar moieties (Boll *et al.*, 2015; Marco-Urrea *et al.*, 2015). Such metabolites display radically  
435 different behaviors compared to the parent PAHs, and tend to partition into the water phase at much  
436 higher rates, which could promote their absorption by micro-organisms and facilitate their transport  
437 through the cell membrane.

#### 438 **4.4 Fungal access to benzo[a]pyrene in compartmentalized environments**

439 Taken together our results enable to propose a two-steps mechanism for the access to BaP by a non-  
440 ligninolytic fungus before its uptake and intracellular biodegradation in a compartmentalized  
441 microenvironment like soil. Firstly, a distant uptake was shown, which highlights an extracellular  
442 mobilization step possibly related to the surface-active agents detected in filtrates or to extracellular  
443 enzyme activities. This first step may increase the presence of BaP or associated hydrophobic  
444 compounds in the water phase and create gradients susceptible to drive growth to the pollutant  
445 source. Secondly uptake appears to be more efficient after the mycelium touches a BaP deposit ,  
446 which could be explained by a better access to the pollutant through a direct chemical partitioning  
447 between BaP crystals and hydrophobic portions of the hyphae surfaces as suggested by the  
448 simulation. These mechanisms of mobilization and uptake could vary according to the  
449 physicochemical properties of the considered PAH and in particular its water solubility. Indeed the  
450 phenanthrene and BaP uptakes by *F. solani* showed different kinetics and were suggested to occur  
451 through different mechanisms (Fayeulle *et al.*, 2014). The better understanding of these active  
452 phenomena to reach hydrophobic pollutants is of relevance to enhance mycoremediation efficiency  
453 though biostimulation approaches, since low PAH bioavailability remains one of the major limitations

454 for the development of these techniques (Akhtar and Mannan, 2020). The elucidation of the  
455 mechanisms by which filamentous fungi influence PAH bioavailability in soils (Posada-Baquero *et al.*,  
456 2019) and transport these molecules through the hyphal network (Harms *et al.*, 2011) are also  
457 important to understand how they can positively interact with other types of organisms within  
458 combined bioremediation strategies (Baranger *et al.*, 2021). Notably positive results in  
459 bioremediation of PAH impacted soils have been obtained through combination of mycoremediation  
460 and phytoremediation (Ma *et al.*, 2021).

## 461 **5 Conclusion**

462 A compartmentalized microchip was designed to observe the incorporation of benzo[a]pyrene by the  
463 fungus *Talaromyces helicus* in a controlled geometry. This is the first time to our knowledge that BaP  
464 fungal uptake is directly observed in a compartmentalized system and that a combination of  
465 extracellular surfactants and direct contact uptake is proposed to explain this phenomenon. Indeed  
466 the fungus is able to develop a mycelium in the confined chambers, and hyphae can grow in direct  
467 contact with BaP crystals. Observations through epifluorescence and confocal microscopy confirmed  
468 that the pollutant is incorporated into the cells of *T. helicus*, and co-staining experiments with BODIPY  
469 lipid dye show that lipid bodies are a preferential storage site in the cell. BaP uptake in *T. helicus*  
470 occurred even when hyphae were not directly touching the crystals, but to a lower extent than when  
471 hyphae were in direct contact with the crystals. Additionally, *T. helicus* produces extracellular surface-  
472 active compounds that were detected in culture filtrates. Thus, the use of a microfluidic device in our  
473 study brings first pieces of evidence for a two-steps mechanism involved in the access to BaP by a  
474 non-ligninolytic soil fungus before its uptake and intracellular biodegradation.

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483        **8 Bibliography**

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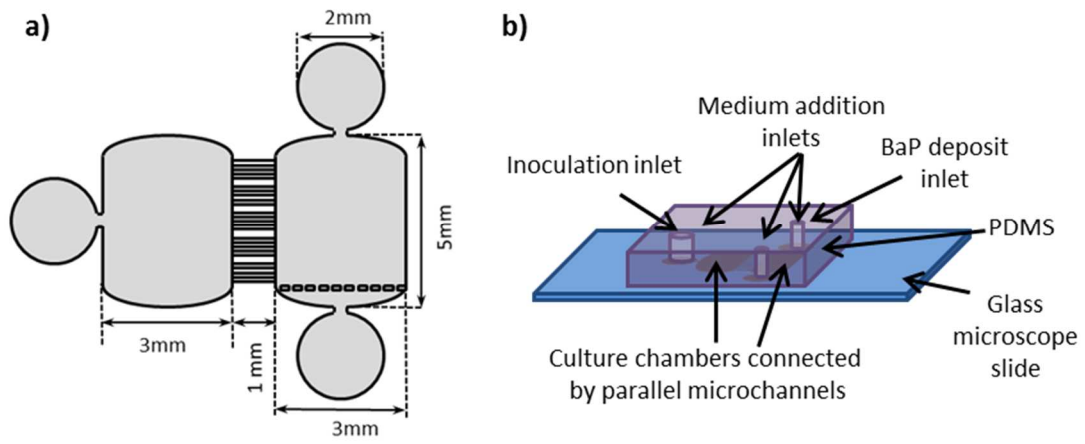
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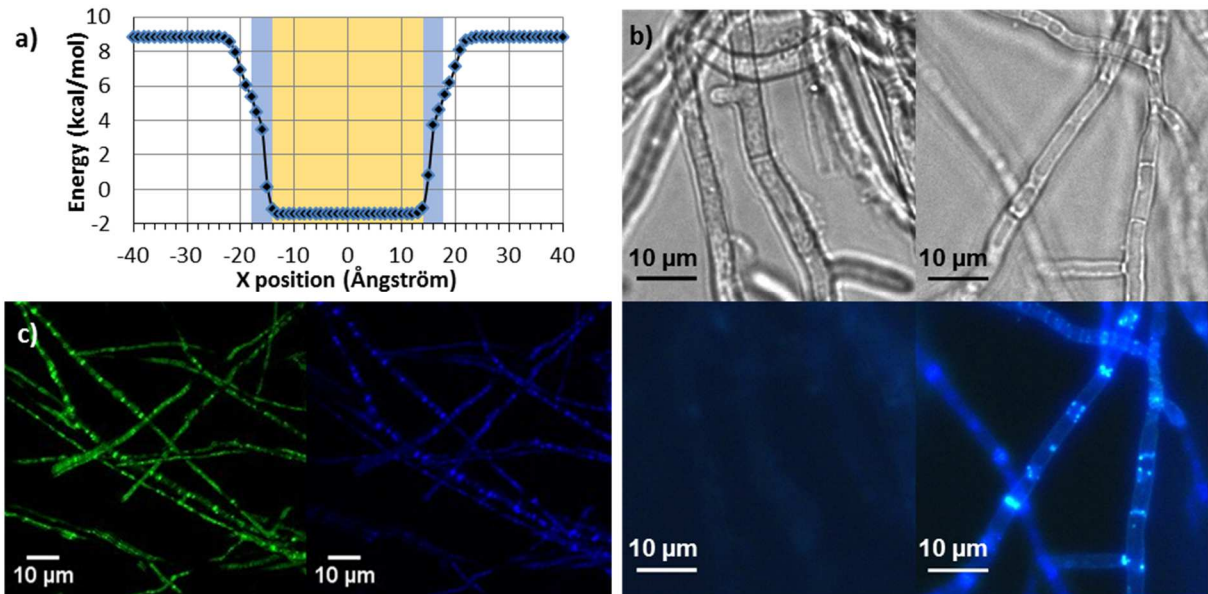
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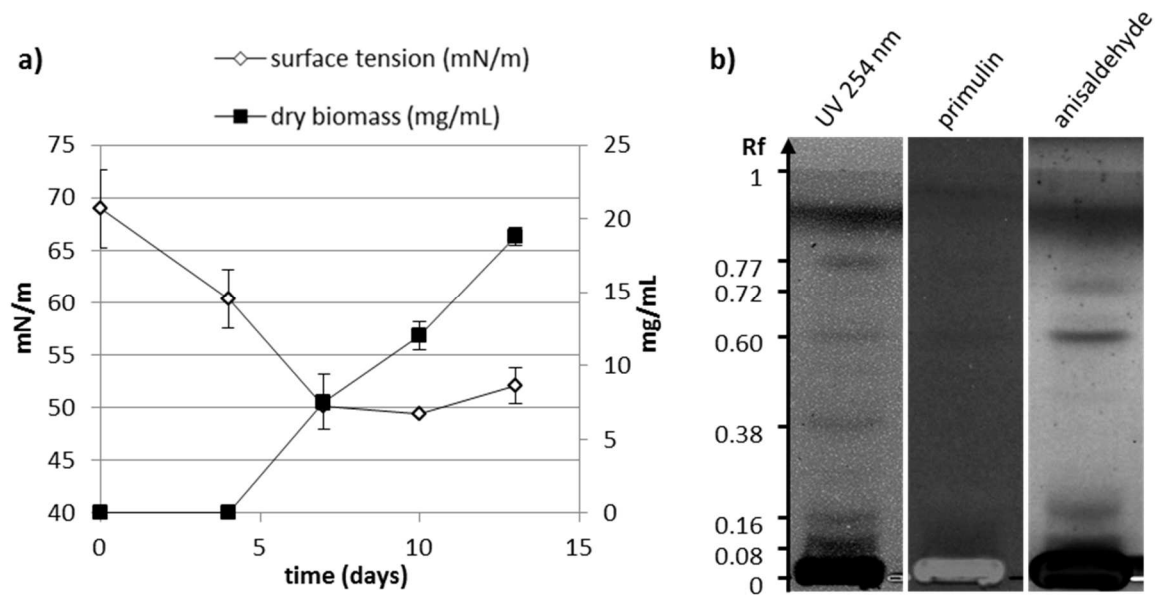


**Fig. 1: Diagram representing the microfluidic device used for fungal cultivation in presence of BaP.**  
**a. channel geometry. b. experimental set-up.**

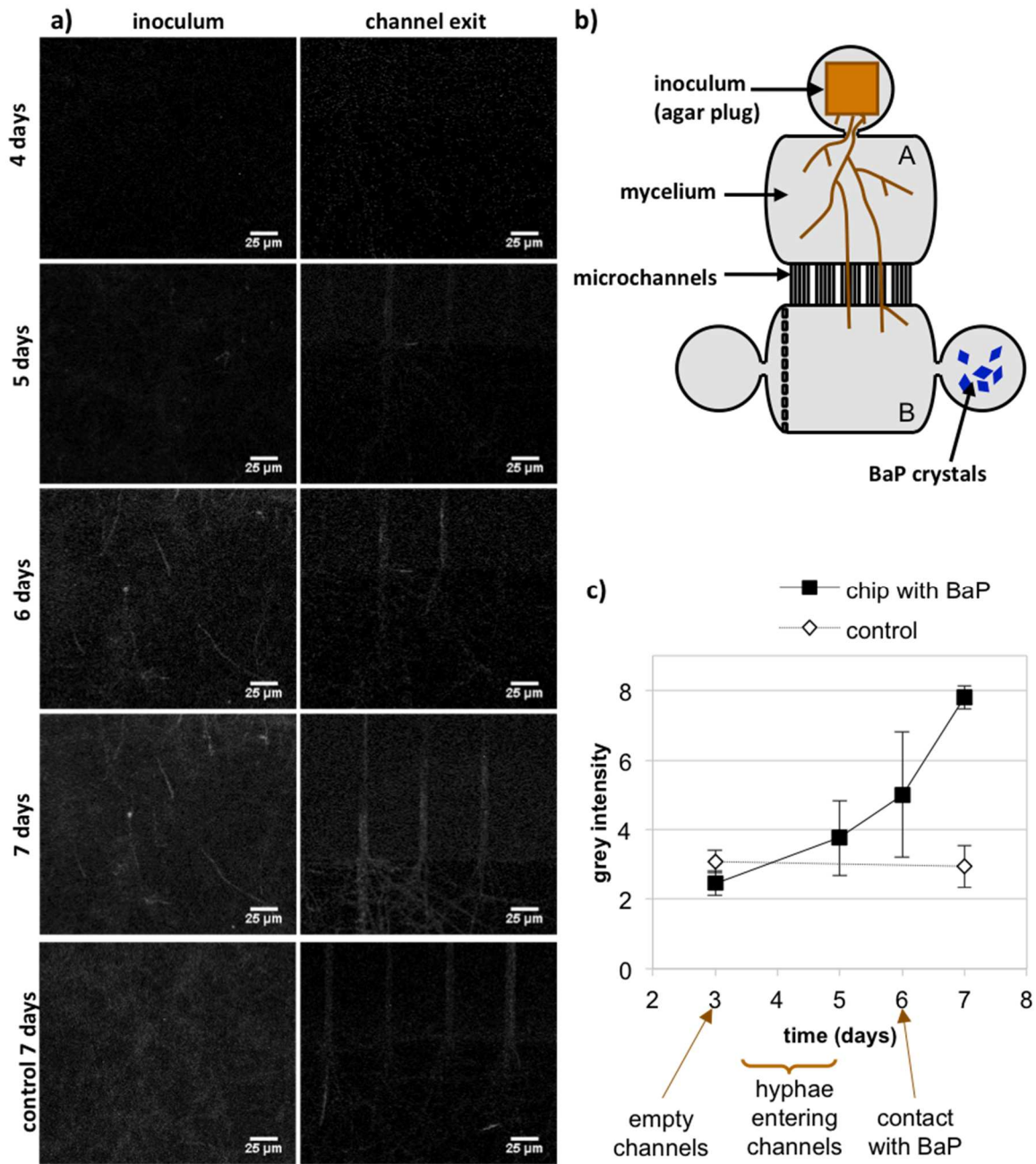


**Fig. 2 :** a - Simulation of BaP penetration across a model lipid bilayer using the IMPALA method. X-axis: position of the mass center of BaP relative to the center of the lipid bilayer; Y-axis: interaction energy calculated as described in Methods. The different regions of the membrane are highlighted in yellow (core hydrocarbon chains) and blue (polar heads). b - Mycelium of *T. helicus* after 24 h of incubation with or without BaP, observed in bright field (top row) and epifluorescence using the DAPI excitation filter (bottom row). left: control mycelium without BaP, showing a faint cell wall autofluorescence; right: mycelium incubated with BaP, displaying small stained vesicles localized at the periphery of hyphal segments. c - Mycelium of *T. helicus* incubated with BaP and stained with BODIPY, observed in confocal microscopy at 40 x magnification (maximum intensity projection of a 41 images stack with a 0.38  $\mu\text{m}$  increment, false colors). Left: FITC filter (green); right: DAPI filter (blue). Both color channels show fluorescent staining colocalized in intracellular vesicles.

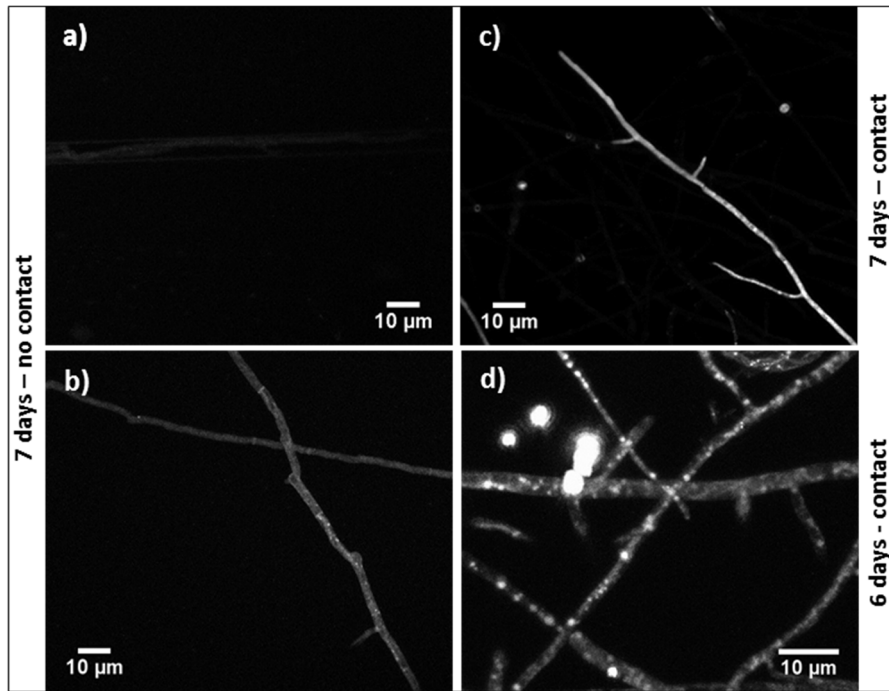




**Fig. 3:** Detection of surface-active compounds in culture supernatants of *T. helicus* grown in plain mineral medium. **a** - surface tension of the cell-free supernatant and dry fungal biomass over time. **b** – HPLC analysis of a 13-day supernatant sample. From left to right: observation under UV light at 254 nm to reveal organic compounds, after primulin staining to detect lipids at 366 nm, and after anisaldehyde staining to detect carbohydrates under white light.



**Fig 4 :** a. Mycelium of *T. helicus* growing in a microfluidic chip spiked with BaP, observed in LSCM at 20x magnification (maximal intensity projection of image stacks with a 1  $\mu$ m increment). Left column: Images taken in chamber A (inoculum). Right column: Images taking at the opening of microchannels on the side of chamber B (BaP deposit). b. Diagram representing the microfluidic device used for fungal cultivation in presence of BaP. c. Variation of fluorescence intensity over time, as represented by the average grey intensity measured in three microchannels in the same microchip over a 10x100  $\mu$ m area (error bars represent the standard deviation of each triplicate; arrows indicate the grey intensity of empty channels, the hyphae entrance in channels and the time after which the direct contact between mycelium and BaP occurred in the considered chip).



**Fig. 5: Mycelium of *T. helicus* growing in microfluidic chips spiked with BaP, observed in LSCM at 40x magnification after 6 to 7 days of incubation. (maximum intensity projection of image stacks with a 1 µm increment). a. Detail of hyphae growing in a microchannel before the mycelium reached the BaP deposit – b. Detail of hyphae growing in chamber B. after the mycelium grew through the channels but not yet reached the BaP deposit. c. Stained hyphal tips in chamber B, close to the BaP deposit. d. Hyphae displaying stained intracellular lipid bodies in chamber B, in direct contact with BaP crystals.**

