Environ Sci Pollut Res (2016) 23:20985–20996
DOI 10.1007/s11356-016-7257-1

RESEARCH ARTICLE

Exploring the potential of fungi isolated from PAH-polluted soil as a source of xenobiotics-degrading fungi

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Received: 14 March 2016 / Accepted: 14 July 2016 / Published online: 3 August 2016

Abstract The aim of this study was to find polycyclic aromatic hydrocarbon (PAH)-degrading fungi adapted to polluted environments for further application in bioremediation processes. In this study, a total of 23 fungal species were isolated from a historically pyrogenic PAH-polluted soil in Spain and taxonomically identified. The dominant groups in these samples were the ones associated with fungi belonging to the *Ascomycota* phylum and two isolates belonging to the *Mucoromycotina* subphylum and *Basiodiomycota* phylum. We tested their ability to convert the three-ring PAH anthracene in a 42-day time course and analysed their ability to secrete extracellular oxidoreductase enzymes. Among the 23 fungal species screened, 12 were able to oxidize anthracene, leading to the formation of 9,10-anthraquinone as the main metabolite, a less toxic one than the parent compound. The complete removal of anthracene was achieved by three fungal species. In the case of *Scopulariopsis brevicaulis*, extracellular enzyme independent degradation of the initial 100 μM anthracene occurred, whilst in the case of the ligninolytic fungus *Fomes* (*Basidiomycota*), the same result was obtained with extracellular enzyme-dependent transformation. The yield of accumulated 9,10-anthraquinone was 80 and 91 %, respectively, and *Fomes* sp. could slowly deplete it from the growth medium when offered alone. These results are indicative for the effectiveness of these fungi for pollutant removal.

Keywords Polycyclic aromatic hydrocarbons (PAHs) · Filamentous fungi · Anthracene · Polluted soil · *Ascomycota* · Biodegradation

Editorial Responsible: Robert Duran

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Introduction

Soil pollution by polycyclic aromatic hydrocarbons (PAHs), which are the main pollutants responsible for air, soil and groundwater contamination, is, since long, one of the major concerns with regard to the impact of anthropogenic activities. In addition to their anthropogenic origin, PAHs are also formed in natural processes such as natural combustion. Structurally, PAHs are formed by the fusion of three or more benzene rings, thus decreasing their bioavailability and biodegradability from low (3–4 rings) to high molecular weight (4–7 rings). They are on the priority list of hazardous substances compiled by the US Environmental Protection Agency (EPA) and regulated by the European Parliament's (2013) Directive 2013/39/EU, due to the high risk for human health and ecosystems.

Microorganisms are key players involved in the biotransformation of xenobiotics in soils, sediments and water bodies. For this reason, the interest in studying the biodegradation of pollutants by microorganisms in contaminated environments, and in creating a successful microbial inoculum for bioremediation purposes has increased, especially in autochthonous organisms already adapted to polluted environments (Cerniglia and Sutherland 2010; Marco-Urrea et al. 2015; Aranda 2016). Several bacteria and fungi are able to biologically degrade PAHs through biotransformation or

mineralization to obtain carbon and energy for the cell metabolism (Keck 1989).

There are a large number of studies on the bioremediation capabilities of fungi and their ability to transform PAHs. However, despite the well-documented fungal presence in highly polluted soils, and although microorganisms may account for up to 75 % of soil biomass, there are very few studies that deal with indigenous fungi in PAH-contaminated soil or the mechanisms and pathways involved in this conversion, which altogether constitutes a black-box approach to real bioremediation processes (Ritz and Young 2004). PAH-polluted soils are usually characterized by limited microbial diversity and a limited overall number of indigenous fungi. These fungi are usually highly tolerant under such conditions, probably as a result of high selective pressure, and they can constitute a powerful tool for pollutant bioconversion (Margesin and Schinner 2001).

Most fungal pollutant degraders belong to the subkingdom *Dikarya* (*Ascomycota* and *Basidiomycota*) and, to a lesser extent, the subphylum *Mucoromycotina* (including the former *Zygomycetes*) (Hibbet et al. 2007; Harms et al. 2011). White rot fungi are well known to be able to convert PAHs through their ligninolytic enzymatic system (Cerniglia 1993). In the case of non-ligninolytic fungi, some species of the genera *Cunninghamella* (order *Mucorales*), *Aspergillus* and *Penicillium* (order *Eurotiales*) have been found to be capable of degrading aromatic compounds, aliphatic hydrocarbons and PAHs through membrane-bound P450 monooxygenases (Hofrichter and Scheibner 1993; Cerniglia 1997; Pinedo-Rivilla et al. 2009; Marco-Urrea et al. 2015). However, other phyla such as *Dothideomycetes* and *Saccharomycetes*, which are more common inhabitants of polluted soils, have been poorly studied as degrading microorganisms.

The isolation of cultivable fungi from polluted soils represents an appropriate alternative that provides fungal strains which are already adapted to polluted conditions and can offer advantages over strains directly obtained from culture collections. Although culture-based techniques have some limitations, these methods remain essential for providing specific living strains for in vitro experimentation (Bull 2004).

This study aimed to isolate and characterize filamentous fungi from a PAH-polluted soil in order to determine whether these fungi have the ability to degrade anthracene, a low molecular weight PAH. These fungi are, therefore, likely to be useful in current industrial bioremediation processes.

Materials and methods

Sampling

The soil samples were collected from an area located in Malaga, Spain (36° 41′ 12.2208″, − 4° 27′ 57.0312″). This soil has historically been contaminated with PAHs due to fuel spills caused by industrial activity in the area. The samples were randomly collected from the top soil (3–6 cm) and immediately used for fungal isolation and chemical analysis of pollutants by organic solvent extraction and subsequent analysis by gas chromatography-mass spectrometry (GC-MS).

Fungal isolation

The fungi were isolated using the soil dilution plate method (Waksman 1922). One gram of soil was mixed with 10 mL sterilized distilled water to form an aliquot; 1/1000 dilutions were then prepared and inoculated on malt extract agar (MEA) dishes with streptomycin and tetracycline as antibiotics (50 and 25 μg mL^{-1}, respectively). After incubation at 26 °C for 3 days, distinct morphotypes were isolated from the diluted solutions grown on the plates.

Individual isolated strains were cultured on MEA medium and incubated at 26 °C for 7 days. The cultures were stored at room temperature, sub-cultured once a month and stored in glycerol. They were then deposited in the culture collection of the Estación Experimental del Zaidín (EEZ-CSIC).

Molecular identification of isolates

Fungal DNA extraction procedure

All the different isolated fungi were grown in MEA dishes. Approximately 1 cm^2 of fungal mycelia was transferred from a fresh dish to a 1.5-mL sterile Eppendorf tube and frozen at −80 °C overnight. Total genomic DNA from the different fungi was isolated, as previously described in Cueva et al. (2011). DNA extracts were re-suspended in 50 μL of Milli-Q water and stored at 4 °C for further use.

PCR amplification

The extracted fungal DNA was used for PCR amplification of the ITS1-5.8S-ITS2 region of the different fungal isolates by using primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al. 1990). Fifty-microliter PCR reactions were prepared containing 50 ng of the extracted fungal DNA, 0.1 mM of each dNTP (Roche), 1 μM of each primer and 0.5 units of Taq DNA polymerase (BIOTAQ™ DNA Polymerase, Bioline), with the appropriate reaction buffer. Amplification was carried out using the following protocol: 1 cycle at 95 °C for 5 min, 40 cycles of 1 min at 95 °C, 1 min at 55 °C and 1.5 min at 72 °C and a further cycle at 72 °C for 7 min. PCR products were electrophoresed in 1.0 % (*w*/*v*) agarose gels (Seakem® LE Agarose; Cambrex Bio Science) and purified using the Illustra™ GFX™ PCR DNA purification kit (GE Healthcare).

Cloning

Products resulting from PCR and containing fragments of the ITS1-5.8S-ITS2 region were cloned into the pGEM-T® vector (Promega) following the manufacturer's instructions. The yielded plasmids were used for sequencing.

DNA sequencing and further analysis

Amplified and cloned DNA fragments were sequenced by using a 16-capillary 3130 XL Genetic Analyzer (Applied Biosystems) available at the Technical Instruments Services of the Estación Experimental del Zaidín. The BLAST software tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare nucleotide acid sequence similarities with the ITS1-5.8S-ITS2 sequences of fungal species deposited in GenBank.

Anthracene biodegradation

Firstly, a tolerance test was carried out in MEA surface culture in 9-cm-diameter glass Petri dishes. Anthracene (5 mM in acetone) was added to the dishes, corresponding to a final concentration of 1 mM, and the solvent evaporated. The capability of the fungi to form radial growth was determined by macroscopic observation.

The isolated fungi were used to detect their capacity to convert PAHs with anthracene as the target compound. Fungal cultivation was carried out in 100-mL Erlenmeyer flasks containing 25 mL of a soybean flour suspension (20 g mL^{-1}). The flasks were inoculated with the contents of the agar of a 9-cm-diameter Petri dish and homogenized in 80 mL sterile water (8 %, *v*/v). After 3 days of incubation, anthracene (10 mM in acetonitrile) was added to the cultures to obtain a solution corresponding to a final concentration of 100 μM (20.3 mg mL^{-1}) (Aranda et al. 2009). As a negative control, we used heat-inactivated mycelium, in order to control (and subtract) the adsorption processes. All cultures were agitated on a rotary shaker at 120 rpm and 28 °C. Three flasks with fungal cultures and three flasks with the respective controls were harvested every 14 days until the end of the experiment on day 42. Manganese-dependent peroxidase (MnP), laccase and aryl alcohol oxidase (AAO) activities as well as pH and mycelium growth were measured in the samples, as described below. Fungal biomass was calculated as the constant weight of the mycelium at 100 °C. The remaining culture liquid was diluted with ethanol (96 %, *v*/v) for anthracene detection by HPLC, as described previously (Steffen et al. 2002). The depletion of 9,10-anthraquinone from the fungal growth medium was carried out accordingly. All experiments were carried out in triplicates, and the data reported are mean values with standard deviations.

Chromatographic analyses

Soil analyses

We carried out a qualitative and a semi-quantitative estimation of soil PAH content using GC-MS in comparison with standards (DRH-008S-R2 for the aliphatic fraction and PAH-Mix9 for the aromatic fraction; Sigma-Aldrich). Analyses were carried out at the Technical Instruments Services of the Estación Experimental del Zaidín. The results from the extraction protocol for hydrocarbons from soil, performed according to Acosta-González et al. (2013), contained both aliphatic and aromatic fractions. Two extraction methods were used: with organic solvents followed by SPE (LC18 Supelco) and by solid-phase microextraction (SPME) directly from the soil. GC-MS analyses were performed using a Varian 450-GC gas chromatograph equipped with a Varian 240 IT mass-selective detector and fitted with a split/splitless injector for a Factor Four VF-5 MS. Separation was carried out using the fused silica capillary column Supelco SLB5-MS (30 m × 0.25 mm × 0.25 μm). The injector was set at 300 °C, and helium (99.999 %) was used as a collision gas in the ion trap chamber and as a carrier gas at a constant flow rate of 1 mL min^{-1}. For the aliphatic fraction, the following oven temperature program used was: 50 °C (5 min) increased by 15 °C min^{-1} to 150 °C and increased by 6 °C min^{-1} to 300 °C (maintained for 10 min). For the aromatic fraction, 1 μL was injected in splitless mode under the following conditions: 50 °C (5 min) increased by 15 °C min^{-1} to 150 °C and then increased by 6 °C min^{-1} to 300 °C (maintained for 10 min).

The headspace (HS) SPME extractions were performed by using 0.1 g soil and 10 mL Milli-Q water. The SPME equilibrium was conducted by immersing the fibre in the headspace of the sample by stirring at room temperature for a period of 40 min.

After extraction, the fibre was thermally desorbed for 5 min into the glass liner of the GC injector port at 280 °C (100-μm PDMS fibres) in splitless mode. The oven temperature program used was as follows: 60 °C, raised by 7 °C min^{-1}, to 130 °C, raised by 5 °C min^{-1}, to 200 °C, 6 °C min^{-1}, to 260 °C and 20 °C min^{-1} to 320 °C (maintained for 4 min). Identification and quantification were performed in single-ion monitoring (SIM) mode using the most abundant ions.

In vivo conversion analyses

Chromatographic analyses of the residual anthracene in the fungal culture media, previously diluted in ethanol, were performed using a HP 1050 HPLC system (Agilent®, Waldbronn, Germany) equipped with a diode array detector (DAD; 190–700 nm). Separations were carried out on a Waters Nova Pack C18, 4 μm reversed-phase column

(150 × 3.9 mm). Acetonitrile and phosphoric acid (15 mM; pH < 2) in water were used in linear gradient elution mode, starting with 30 % acetonitrile and reaching 80 % acetonitrile within 10 min. The flow rate was 0.85 mL min^{-1}. Eluted substances were detected in the wavelength range from 210 to 280 nm.

Enzymatic analyses

Enzymatic activities in the different liquid fungal cultures were assessed. MnP activity was specifically assayed, as described previously, by monitoring the formation of the Mn^{3+}–malonate complexes at 270 nm (ε 270 = 11.2 mM cm^{-1}) (Wariishi et al. 1992). Laccase was determined by following the oxidation of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (ε 420 = 36 mM cm^{-1}) in 50 mM sodium citrate buffer at pH 4.5 (Eggert et al. 1995). The reaction was started by the addition of 1 mM H_2O_2. Using the method described by Muheim et al. (1990), aryl alcohol oxidase (AAO) was assayed by measuring the oxidation of veratryl alcohol at 310 nm (ε 310 = 9.3 mM cm^{-1}) using a potassium phosphate buffer at pH 6.0.

The enzyme activity was expressed as unit per mL^{-1}, with a unit being defined as the amount of enzyme that catalyzes the conversion of 1 μmol of substrate per minute. In all cases, the mean values of triplicate determinations were calculated.

Chemicals

Anthracene (99 % purity), anthrone (98 % purity) and 9,10-anthraquinone (98 % purity) were purchased from Sigma-Aldrich (Madrid, Spain). HPLC-gradient grade acetonitrile and ethanol (96 %) were obtained from VWR International (Barcelona, Spain).

Statistical analyses

All experiments were conducted in triplicates. All data were plotted and analyzed for statistical significance by using Statgraphics Centurion XVI.I software. The data were compared by using one-way analysis of variance (ANOVA) and the Duncan's post test.

Results

Soil analyses

The results obtained by the HS-SPME-GC/MS analyses showed that the soil used in this study was mainly contaminated with two- and three-ring aromatic hydrocarbons, a large proportion of which being naphthalene, anthracene and phenanthrene. Quantitative analyses of these compounds by GC-MS showed amounts of 0.16 ± 0.009, 0.05 ± 0.021 and 0.052 ± 0.016 ppm, respectively, with phenanthrene being eight-fold higher than the EPA regulation limit (0.0062 ppm). Oxidized anthracene and methylated naphthalenes, such as methylnaphthalenes, were also found (Table 1). In addition, the presence of sulphur in aromatic compounds, such as the plasticizer N-butylbenzenesulfonamide, was detected. Since the bioavailabilities and mobilities of PAHs are greatly affected by soil organic matter content, the total organic carbon (TOC) was measured, yielding 11.2 ± 1.13 per 100 g soil (estimated at the Ionomics Service of CEBAS-CSIC).

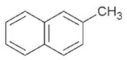
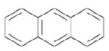
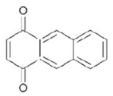
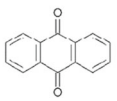
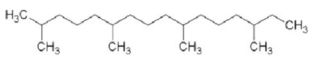
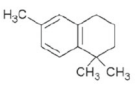
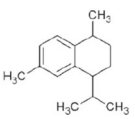
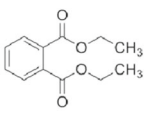
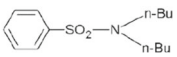
Isolation of fungi

A total of 23 soil fungi were isolated from the selected polluted area. In the first screening, involving the growth of the isolated fungi in agar Petri dishes containing anthracene in MEA, we found that all of the fungi were tolerant species towards this compound (data not shown). From each of them, the amplified and cloned transcripts corresponding to the ITS1-5.8S-ITS2 region of each cultivable fungal strain were sequenced and genetically identified by comparison with other ITS1-5.8S-ITS2 sequences stored in available databases. BLAST analyses showed that all the sequences of the positive ITS amplicons met the stringent sequence quality (>80 % quality score) and blast match affinity (>95 % max identity) criteria (Table 2). This led to the identification of 16 different fungal species and 7 genera with genetic similarity values of close to 100 % as compared with those reported in GenBank (Table 2). The taxonomic distribution of the isolated fungal species from soil is shown in Fig. 1a. In this soil, the most abundant classes of fungi were *Eurotiomycetes* (40 %), *Dothideomycetes* (26.6 %) and *Sordariomycetes* (20 %), all of which belong to the subphylum *Pezizomycotina* and the phylum *Ascomycota* (Fig. 1a). We also found that between 3 and 6 % of the species belonged to *Saccharomycotina*, *Mucoromycotina* and *Agaricomycotina*. The most frequently isolated species (largest number of isolates) were *Exophiala, Trichoderma* and *Aspergillus* (Table 2). The phylogram in Fig. 1b shows that the 23 strains can be assigned to 16 species and 5 genera belonging to the *Ascomycota* phylum (21 strains), the *Basidiomycota* phylum (one strain) and the *Mucoromycotina* subphylum (one strain). Most genera were represented by a single strain, with the exception of *Trichoderma* (two strains), *Aspergillus* (two strains) and *Exophiala* (four strains) (Table 2).

Anthracene conversion

In the batch experiments performed in liquid cultures using soybean flour as an additional carbon source, only 12 fungi were able to convert the anthracene at different rates (Fig. 2a):

Table 1 Qualitative analysis of the contaminated soil by HS-SPME-GS-MS. All shown compounds were found in the sample

PAHs	Chemical structure	Molecular Formula	MW (g mol^{-1})
Naphthalene		$C_{10}H_8$	128.17
2-Methylnaphthalene		$C_{11}H_{10}$	142.20
1-Methylnaphthalene		$C_{11}H_{10}$	142.20
Anthracene		$C_{14}H_{10}$	178.23
1,4-Anthraquinone		$C_{14}H_8O_2$	208.21
9,10-Anthraquinone		$C_{14}H_8O_2$	208.21
Phenanthrene		$C_{14}H_{10}$	178.23
Aliphatic			
nC_{18}			
nC_{17}			
Phytane		$C_{20}H_{42}$	282.56
Other pollutants			
1,1,6-Trimethyltetraline		$C_{13}H_{18}$	174.28
1,2,3,4-Tetrahydro-1,6-dimethyl-4-(1-methylethyl)- (1S-cis) naphthalene		$C_{15}H_{22}$	202.33
1,2,3-Trimethylbenzene		C_9H_{12}	120.19
Sulphur		S	32.07
Diethyl phthalate		$C_{12}H_{14}O_4$	222.24
N,N-Di-n-Butylbenzenesulfonamide		$C_{14}H_{13}NO_2S$	259.32

Drechslera phlei (Fig. 2a), *Scopulariopsis brevicaulis* (Fig. 2b), *Trichoderma* sp. (Fig. 2c), *Trichoderma harzianum* (Fig. 2d), *Aspergillus niger* (Fig. 2e), *Fomes* sp. (Fig. 2f), *Aspergillus ustus* (Fig. 2g), *Ophiosphaerella agrostidis*

Table 2 Identity of fungi isolated from the polluted soil based on the closest blast match of fungal ITS sequences. Query sequences were considered to match reference sequences in GenBank only if the max identity score was >95 %

Isolate ID	Phylum class	Closest blast match		
		Strain	GenBank accession no.	% Identity
1	*Dothideomycetes*	*Drechslera phlei*	JN940094	100
2	*Sordariomycetes*	*Scopulariopsis brevicaulis*	KC311514	100
3	*Sordariomycetes*	*Hypocrea jecorina*	AF10497	99
4	*Sordariomycetes*	*Trichoderma sp.*	GQ169750	100
5	*Sordariomycetes*	*Trichoderma harzianum*	KC330218	99
6	*Eurotiomycetes*	*Aspergillus niger*	AB363747	100
7	*Agaricomycetes*	*Fomes sp.*	HM136673	100
8	*Eurotiomycetes*	*Exophiala sp.*	AF050267	99
9	*Saccharomycetes*	*Candida davisiana*	GU373794	99
10	*Dothideomycetes*	*Glonium pusillum*	EU552134	99
11	*Eurotiomycetes*	*Exophiala phaeomuriformis*	JX669018	99
12	*Eurotiomycetes*	*Exophiala dermatitidis*	AY213650	99
13	*Mucoromycotina*	*Mucor circinelloides*	DQ118990	99
14	*Sordariomycetes*	*Podospora sp.*	AY345351	99
15	*Eurotiomycetes*	*Aspergillus ustus*	AY216676	99
16	*Dothiodeomycetes*	*Ophiosphaerella agrostidis*	KM434282	99
17	*Eurotiomycetes*	*Exophiala sp*	AF050267	99
18	*Eurotiomycetes*	*Penicillium pinophilum*	HM469418	100
19	*Dothideomycetes*	*Loratospora aestuarii*	GU301838	99
20	*Dothideomycetes*	*Pleosporales sp.*	HQ649862	99
21	*Dothideomycetes*	*Leptosphaeria proteicola*	JQ044458	99
22	*Dothideomycetes*	*Lewia infectoria*	AY154691	99
23	*Dothideomycetes*	*Cladosporium cladosporioides*	JN651416	99

(Fig. 2h), *Penicillium pinophilum* (Fig. 2i), *Loratospora aestuarii* (Fig. 2j), *Pleosporales* sp. (Fig. 2k) and *Lewia infectoria* (Fig. 2l). The most efficient anthracene degraders were *S. brevicaulis* and *Fomes* sp., which could convert 100 μM of anthracene within 21 days of incubation. The *Pleosporales* sp. strain was able to degrade this amount within 42 days (Fig. 2A). The fungi *T. harzianum*, *A. ustus* and *P. pinophilum* had conversion rates of over 50 % after 21 days of cultivation. However, the fungi *D. phlei*, *A. niger*, *L. aestuarii*, *O. agrostidis* and *L. infectoria* showed conversion rates of less than 50 % after 21 days of cultivation. The biomass content reached 12–15 mg mL^{-1} of most fungi; growth generally decreased after 21 days of cultivation, and the pH of the cultured liquid markedly increased over time. Adsorption of anthracene by the fungal biomass was negligible (less than 0.1 %).

One single metabolite was identified in liquid cultures of these fungi tested after incubation with anthracene at the retention time of 13.7 min (Fig. 3). It corresponded to 9,10-anthraquinone, with no further degradation of the quinone being detected during the time span of the experiment in most of the experimental setups. The identity of this metabolite was confirmed using a corresponding authentic standard by its retention time and UV spectrum (Fig. 3). The conversion of anthracene into 9,10-anthraquinone was very often incomplete, yielding amounts of 75 and 93 μM in the case of *S. brevicaulis* and *Fomes* sp., respectively (Figs. 2 and 3). The rest of the peaks analysed were also detected in the inactivated control of the experiment and attributed to the soybean medium.

In order to verify whether yes or no the accumulated quinone represents a dead-end metabolite or just a transiently accumulating one, we incubated the fungal strain *Fomes* sp. with this compound in the presence of soybean flour and prepared an inactivated control (Fig. 2b). When compared with the latter one where no depletion became detectable, the anthraquinone disappeared slowly in the living cell system, down to 40 % of the initial concentration at day 20.

Enzymatic activity

The most important extracellular enzyme producer was the Basidiomycete *Fomes* sp., which reached 1299 and 207.5 U mL^{-1} of MnP and laccase, respectively, after 21 days of cultivation (Table 3). *O. agrostidis* also showed remarkably high MnP activity of 244 U L^{-1} after 42 days of incubation. The other isolated fungi did not show any significant extracellular enzymatic activity, with the exception of *D. phlei*,

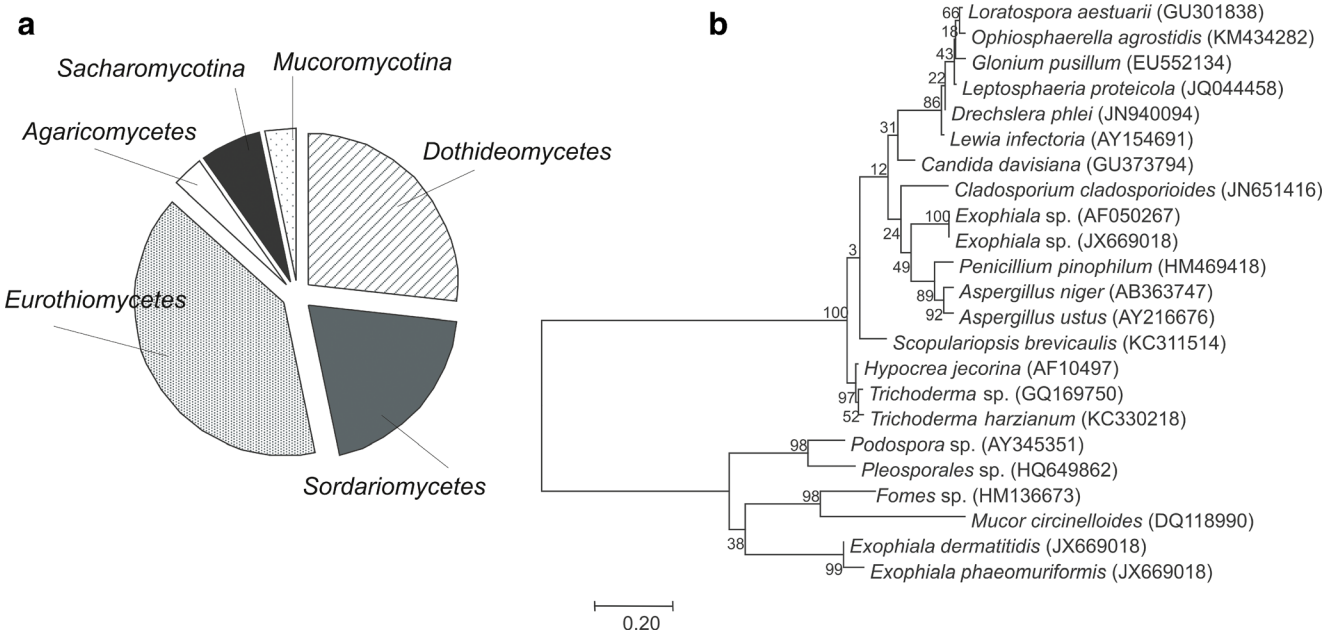


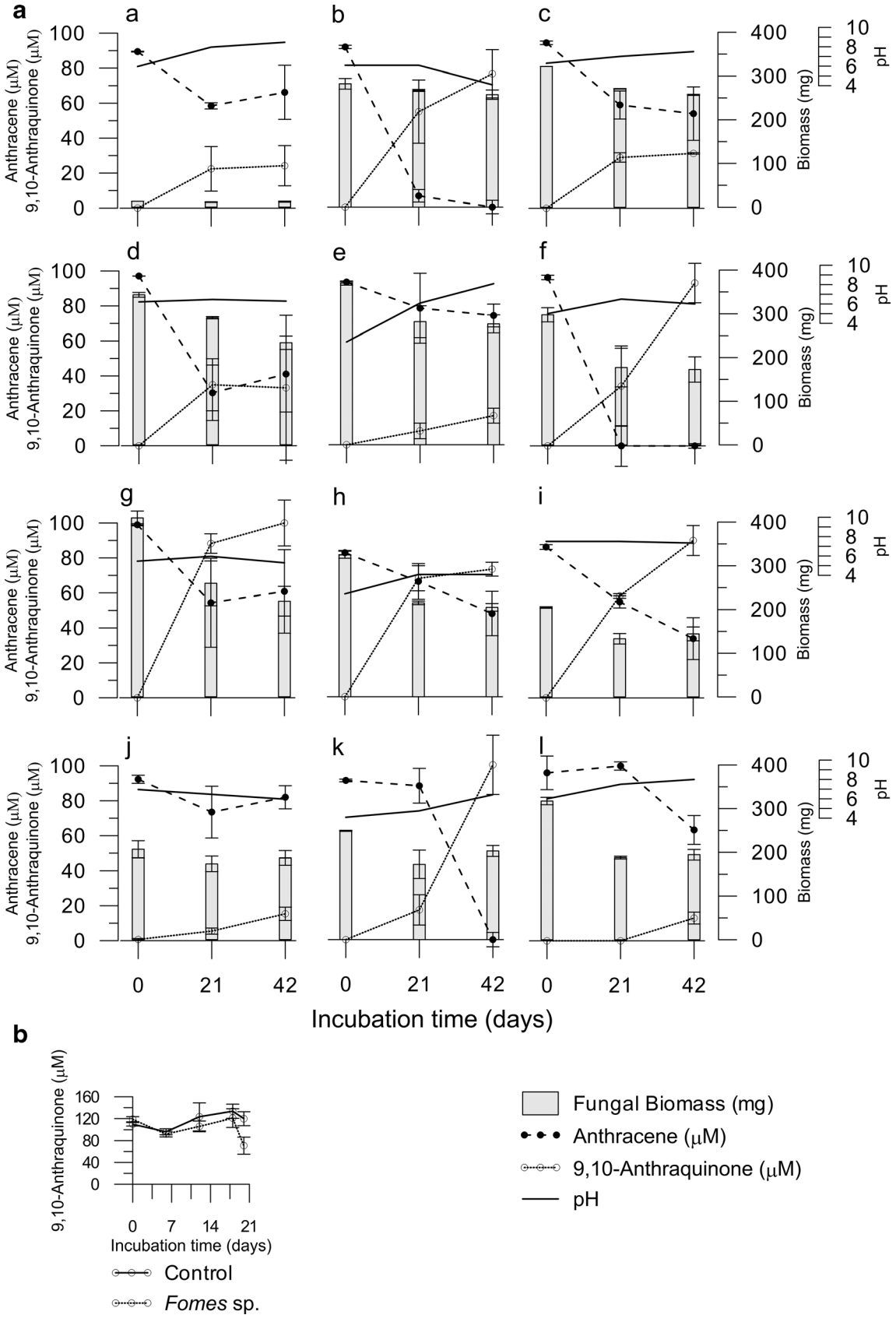
Fig. 1 Phylogenetic distribution of the isolated cultivable fungi from contaminated soil **(a)**. Consensus phylogenetic tree based on partial fungal ITS1, 5.8s rRNA. The evolutionary history was inferred by using the maximum likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). The tree with the highest log likelihood (−4400.7016) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 23 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + noncoding. All positions containing gaps and missing data were eliminated. There were a total of 402 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016) **(b)**

Thrichoderma sp. and *T. harzianum*, *A. niger* and *A. ustus* and *P. pinophilum* and *L. aestuarii*. *D. phlei* showed an increase in MnP and AAO after 21 days of incubation. *A. niger* also showed remarkably high MnP activity after 21 and 42 days of incubation. No extracellular enzymes were detected in cultures of *S. brevicaulis* and *L. infectoria*.

Discussion

In the present study, the isolation of fungal species from a PAH-polluted soil, caused by the successive spill of petroleum-refined products was carried out. This soil showed the presence of species that mainly belonged to the phylum *Ascomycota*, *Mucoromycotina* subphylum (formerly *Zygomycota* phylum) and *Basidiomycota*. *Ascomycota* represents one of the most diverse groups of fungi capable of colonizing almost all ecological niches. Together with the *Mucoromycotina* subphylum, *Ascomycota* is the most common group in contaminated soils (Marco-Urrea et al. 2015). Thus, some examples in creosote or PAH-polluted soils were found using culture-dependent techniques as well as shotgun sequencing approaches (Atagana et al. 2006; Reyes-Cesar et al. 2014; Lladó et al. 2015). Basidiomycete lignin degraders, similar to the only one isolated in our study, are rarely found in these environments and are more typical of wood-decaying conditions. Their abilities to degrade aromatic hydrocarbons such as PAHs or BTEX have also been verified and extensively studied (Bumpus 1985). However, non-ligninolytic fungi have been little studied in this context. The variety of cultivable fungi found in the soil being studied was predominantly made up of *Eurotiomycetes* and *Dothideomycetes*. Most of the isolated fungal species have also been reported in other PAH-polluted soils. For instance, species of the genera *Cladophialophora* and *Exophiala* (order *Chaetothyriales*) assimilate toluene (Prenafeta-Boldú et al. 2006), while species of the genera *Penicillium*, *Aspergillus* and *Trichoderma* colonize typical polluted areas; they are having a high level of tolerance to PAHs and are being able to co-metabolize these PAHs (Hofrichter et al. 1994; Atagana et al. 2006; Pinedo-Rivilla et al. 2009; Mineki et al. 2015). Interestingly, yeasts were not isolated in this study, which is in line with Covino et al. (2016).

Half of the total isolated fungal species were able to transform anthracene efficiently in co-metabolic batch experiments, being *S. brevicaulis* and *Fomes* sp., (*Ascomycota* and *Basidiomycota*, respectively) the most efficient ones. Both fungi transformed anthracene into 9,10-anthraquinone which is one of the first oxidation products of anthracene described in fungi. Its further degradation by the Agaricomycetes *Phanerochaete chrysosporium* (*Basidiomycota*) through the cleavage of the central aromatic ring system to yield phthalate


a
a
b
c
d
e
f
g
h
i
j
k
l
Anthracene (μM)
9,10-Anthraquinone (μM)
Biomass (mg)
pH
0
20
40
60
80
100
0
100
200
300
400
4
6
8
10
0
21
42
Incubation time (days)
b
9,10-Anthraquinone (μM)
0
40
80
120
160
0
7
14
21
Incubation time (days)
Control
Fomes sp.
Fungal Biomass (mg)
Anthracene (μM)
9,10-Anthraquinone (μM)
pH

◀ **Fig. 2** **a** Time course of anthracene conversion by the fungi *a Drechslera phlei*, *b Scopulariopsis brevicaulis*, *c Trichoderma* sp., *d Trichoderma harzianum*, *e Aspergillus niger*, *f Fomes* sp., *g Aspergillus ustus*, *h Ophiosphaerella agrostidis*, *i Penicillium pinophilum*, *j Loratospora aestuarii*, *k Pleosporales* sp. and *l Lewia infectoria*. *Bars* represent fungal biomass (mg mL^{-1}). *Lines* represent anthracene concentration (*dashed line*), 9,10-anthraquinone formation (*dotted line*) and pH (*continuous line*). Data are mean values with standard deviations of triplicate samples. **b** Time course of 9,10-anthraquinone conversion by the fungus *Fomes* sp. *Lines* represent 9,10-anthraquinone concentration in the inactivated fungal control (*dotted line*) and in the living one with *Fomes* sp. (*continuous line*)

was shown, including its at least partial mineralization to CO_2 (Hammel et al. 1991).

Two different mechanisms could be used in this anthracene conversion, since in the case of *S. brevicaulis*, no extracellular enzymes were detected. Thus, the initial stage of fungal attack on these compounds could rely on the intracellular detoxification system for xenobiotics mediated by membrane-bound enzymes, such as cytochrome P450 monooxygenases (CYPs) and epoxide hydrolases, which have been described for non-ligninolytic fungi (Cerniglia and Sutherland 2010). However, since we did not detect hydroxylated or conjugated metabolites, we cannot confirm this pathway.

9,10-Anthraquinone, however, does not always appear to be a dead-end product in these batch experiments when no additional metabolites were found: when we incubate the fungal strain *Fomes* sp. only with this compound, it slowly disappeared (Fig. 2b) Thus, the non-stoichiometric ratio between anthracene eliminated and 9,10-anthraquinone accumulated could indicate that anthracene may have been degraded via another pathway and partially mineralized to CO_2, water and biomass. This way of degradation of aromatic compounds presumably occurs in some of the other fungi studied so far (Bezalel et al. 1996). Other techniques should be applied in order to decipher the complete route. With regard to the lignin-degrading fungus *Fomes* sp., anthracene oxidation occurred probably very slowly via mineralization into carbon dioxide and water, which is a well-known process in white rot fungi (Hammel et al. 1991; Reddy 1995). The high rate of MnP production and the moderate production of laccase appear to be responsible for the high yields of anthracene conversion, indicating that the first step is being mediated by the high level of oxidoreductase enzymes produced. In our case, the main metabolite produced was also 9,10-anthraquinone. *Fomes* sp. has previously been described as an efficient laccase and MnP producer, as well as a degrader of pollutants such as dyes (Ramesh 2009; Větrovský et al. 2013).

The rest of the fungi studied did not show the high extracellular enzymatic activity found in the basidiomycetous

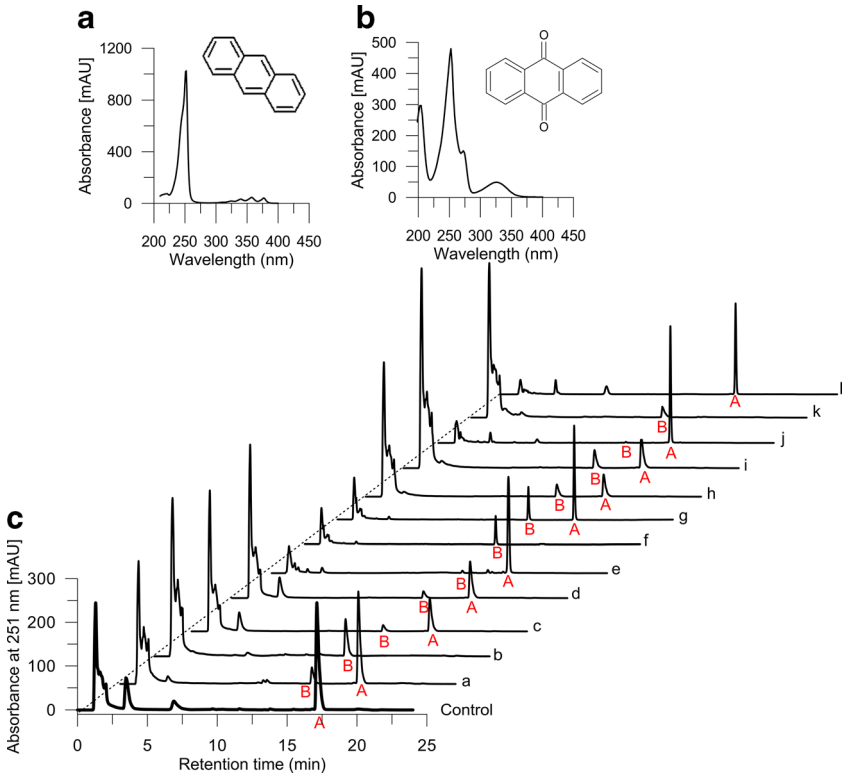


Fig. 3 Panels **a** and **b** represent the UV spectra of anthracene and 9,10-anthraquinone, respectively. **c** The cultivation of the fungal species was performed in soybean flour medium under agitation at 120 rpm at 28 °C. HPLC chromatograms of the culture supernatants were recorded at 251 nm, including an inactivated fungal control, of *a Drechslera phlei*, *b Scopulariopsis brevicaulis*, *c Trichoderma* sp., *d Trichoderma harzianum*, *e Aspergillus niger*, *f Fomes* sp., *g Aspergillus ustus*, *h Ophiosphaerella agrostidis*, *i Penicillium pinophilum*, *j Loratospora aestuarii*, *k Pleosporales* sp. and *l Lewia infectoria*, at 42 days after addition of anthracene

Table 3 Extracellular enzymatic activities, laccase, manganese-dependent peroxidase (MnP), and aryl alcohol oxidase (AAO) produced by the selected cultivable fungi under in vivo culture conditions at 21 and 42 days after the addition of 100 μM anthracene. Data are mean values with ±standard deviation of triplicate samples

	Incubation days	Laccase (U L^{-1})	MnP (U L^{-1})	AAO (U L^{-1})
Drechslera phlei	21	4.27 ± 0.41	nd	47.25 ± 3.89
	42	8.41 ± 0.09	52.60 ± 0,55	149.67 ± 12.67
Scopulariopsis brevicaulis	21	nd	nd	nd
	42	nd	nd	nd
Trichoderma sp	21	2.07 ± 1.29	12.95 ± 8.11	11.67 ± 5.35
	42	0.27 ± 0.38	1.69 ± 2.40	12.35 ± 4.32
Trichoderma harzianum	21	2.74 ± 0.79	17.17 ± 4.98	120.16 ± 54.88
	42	1.02 ± 0.36	6.39 ± 2.27	109.30 ± 5.47
Aspergillus niger	21	5.68 ± 1.13	35.61 ± 7,07	161.31 ± 17.65
	42	14.75 ± 4.20	62.35 ± 26.32	nd
Fomes sp.	21	207.50 ± 32.20	1299.10 ± 110.90	71.23 ± 39.32
	42	67.85 ± 74.35	424.82 ± 45.50	nd
Aspergillus ustus	21	1.02 ± 1.52	13.85 ± 5.34	1.71 ± 0.34
	42	4.14 ± 0.94	23.37 ± 7.65	25.39 ± 10.88
Ophiosphaerella agrostidi	21	2.4 ± 0.90	112.91 ± 32.80	172.6 ± 77.05
	42	0.55 ± 0.24	244.30 ± 29.40	45.56 ± 18.31
Penicillium pinophilum	21	nd	9.50 ± 3.28	7.97 ± 0.71
	42	2.24 ± 1.30	33.80 ± 8.61	23.89 ± 18.04
Loratospora aestuarii	21	4.51 ± 1.50	63.30 ± 21.61	36.5 ± 23.10
	42	0.95 ± 0.92	4.61 ± 4.62	120.8 ± 33.71
Pleosporales sp.	21	2.47 ± 0.52	3.81 ± 1.50	54.40 ± 9.14
	42	26.61 ± 19.36	65.72 ± 38.41	54.91 ± 9.41
Lewia infectoria	21	nd	7.87 ± 1.64	0.73 ± 0.47
	42	0.81 ± 0.80	5.59 ± 3.57	0.77 ± 0.89

nd not detected

Fomes sp.; however, they efficiently oxidized anthracene at diverse rates. *P. pinophilum* has not been described in anthracene bioconversion up to now. 9,10-Anthraquinone was the only metabolite detected in this case with the aid of our analytical techniques used, probably due to the minute presence of MnP in the extracellular medium and, probably, with the help of the intracellular detoxification system for xenobiotics. This participation has been demonstrated in species of *Penicillium* during the conversion of pyrene. This fungus produced pyrenoquinone and metoxy derivatives as the principal metabolites, which are related to the intracellular degradation system for xenobiotics, the CYP system (Wunder et al. 1997). The two *Trichoderma* strains removed anthracene at similar rates in which the AAO and the low MnP levels could have an important role. Experiments performed by Argumedo-Delira et al. (2012) have shown 11 *Trichoderma* fungal strains which are extremely resistant to high PAH doses. In addition, the capability of *Trichoderma* sp. to metabolize phenanthrene and benzo[a]pyrene has been demonstrated, suggesting the participation of some oxidase enzymes (Machín-Ramírez et al. 2010). In the case of *Aspergillus*, *A. ustus* and *A. niger* showed moderate conversion rates for anthracene with the participation of low amounts of peroxidase activities. Some studies using autochthonous species of *Aspergillus* have demonstrated their capability to transform anthracene, phenanthrene and benzopyrene, which involved methyl-transferases and the corresponding formation of methyl derivatives (Capotorti et al. 2005). Further studies are necessary to demonstrate the participation of this intracellular system in anthracene conversion within these fungi.

All of the fungal strains studied in this experiment produced quinone metabolites which, according to Schmidt et al. (2010) and Meulenberg et al. (1997), could be further degraded by other microorganisms in the soil, as they demonstrated in experiments using 9-anthrone and 9,10-anthraquinone.

It is worth noting that some isolated fungi, such as *Scopulariopsis*, *Drechslera* and *Lewia*, capable of transforming anthracene, are considered to be opportunistic pathogenic species for humans. However, to the best of our knowledge, information concerning the degradation capability of these species

for xenobiotics is very scarce. It has been reported that *Lewia* strains can improve pyrene phytoremediation by *Festuca* sp. plants (Cruz-Hernández et al. 2013). However, this is not the first report on the capability of human pathogenic fungi such as *Cladophialophora* and *Exophiala* to degrade aromatic hydrocarbons. Some authors suggest that this primary niche may be hinted by the capability for hydrocarbon degradation of these fungi (Prenafeta-Boldú et al. 2006).

Conclusion

From the results obtained, we can conclude that the ability of adapted fungi to transform the model PAH compound anthracene could be found among several ecological and taxonomic fungal groups, particularly *Ascomycota* fungi, which appear to be key players in PAH-polluted scenarios. They can use pathways that may involve oxidation to oxy-PAHs such as anthraquinone, which could be further utilized by other microorganisms, or lead to complete mineralization. However, since they do not produce high amounts of the classical lignin-modifying enzymes, it is of major importance to study the involved mechanisms for anthracene conversion as well as their capability to degrade other xenobiotic compounds. Why species from different sources remove aromatic contaminants co-metabolically or fortuitously and whether the fungal enzymatic system is subject to selective reinforcement to be able to mineralize anthracene via *ortho*-phthalate and catechol pathways remain unclear. In conclusion, isolated fungi from polluted sources represent an unexplored field of research and are highly suited for bioremediation processes. Respective studies are being carried out since recently in our laboratory in order to fully understand the mechanisms involved in the adaptation to these conditions and the possible mineralization capabilities and to comprehensively describe the metabolic pathways under these conditions.

Acknowledgments This study was supported by the Junta de Andalucía (project P09-CVI-4778). E. Aranda likes to thank the Ministry of Economy and Competitiveness (MINECO) and FEDER funds for co-funding the Ramón y Cajal contract (RYC-2013-12481). We also wish to thank Lourdes López Ruiz for 9,10-anthraquinone pre-experiment, Maria Angeles Delgado for the technical assistance, Juan Cristobal Romero for providing access to the sampling site, and Michael O'Shea for proofreading the document.

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