# Development of eukaryotic zoospores within polycyclic aromatic hydrocarbon (PAH)-polluted environments: a set of behaviors that are relevant for bioremediation

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**Abstract**

In this study, we assessed the development (formation, taxis and settlement) of eukaryotic zoospores under different regimes of exposure to polycyclic aromatic hydrocarbons (PAHs), which imitated environmental scenarios of pollution and bioremediation. With this aim, we used an oomycete, *Pythium aphanidermatum*, as a source of zoospores and two PAH-degrading bacteria (*Mycobacterium gilvum* VM552 and *Pseudomonas putida* G7). The oomycete and both bacteria were not antagonistic, and zoospore formation was diminished only in the presence of the highest bacterial cell density ($10^8$-$10^{10}$ colony-forming units $mL^{-1}$). A negative influence of PAHs on zoospore formation and taxis was observed when PAHs were exposed in combination with organic solutions and polar solvents. Co-exposure of PAHs with non-polar solvents [hexadecane (HD) and 2,2,4,4,6,8,8-heptamethylnonane (HMN)] did not affect zoospore settlement at the interfaces of the organic solvents and water. However, zoospores settled and created mycelial networks only at HD-water interfaces. Both bacteria diminished the toxic influence of PAHs on zoospore formation and taxis, and they did not interrupt zoospore settlement. The results suggest that zoospore development could be applicable for toxicity assessment of PAHs and enhancement of their bioavailability. Microbial interactions during both swimming modes and community formation at pollutant interfaces were revealed as major factors that have potential relevance to bioremediation.

***Keywords:*** Eukaryotic zoospore; Oomycetes; PAH-polluted scenario; PAH-degrading bacteria; Zoospore development; Bioremediation

## 1. Introduction

In nature, free-swimming zoospores are produced through asexual reproduction by various eukaryotic organisms, e.g., algae, protists, fungi and oomycetes. Although these eukaryotic zoospores are produced from very distant phylogenetic taxa, they share a typical behavioral sequence of development, including swimming, settlement, encystment, germination and orientation of the germ-tube (Walker and van West, 2007; Gleason and Lilje, 2009). The development of zoospores is highly dependent on their chemoresponses to different chemical effectors that are found in the environment, which may cause an incomplete development sequence (Jones et al., 1991; Donaldson and Deacon, 1993; Walker and van West, 2007). Zoospore producers have been found in a wide variety of ecological niches, e.g., cattle's rumen, freshwater lakes, mangrove forests and oceans (James et al., 2006; Walker and van West, 2007; Gleason and Lilje, 2009). Within such ecological niches, they may play diverse roles as symbionts, phototrophs, saprophytes and parasites.

The ecological impact of zoospores and their development have been studied primarily in natural habitats or under axenic conditions (Gleason and Lilje, 2009; Savory et al., 2014), which are rarely observed within polluted environments. Some authors recently reported that mycelial networks formed by a zoospore producer, *Pythium ultimum*, facilitated the transport of either polycyclic aromatic hydrocarbons (PAHs) or PAH-degrading bacteria, thus enhancing the bioavailability of PAHs (Wick et al., 2007; Furuno et al., 2010; 2012). This zoospore producer is a rhizosphere oomycete that belongs to the pseudofungi that live at the interface of biphasic habitats (solid-liquid, solid-air or liquid-air). Although mycelial networks of the oomycete are applicable for enhancement of pollutant bioavailability, the development of its

zoospores in contact with pollutants and pollutant-degrading bacteria still remains relatively unexplored.

Among hazardous chemicals, PAHs are ubiquitous and considered important pollutants that are of critical concern for animal and human health (Keith and Telliard, 1979; Ortega-Calvo et al., 2013). Emissions of PAHs into the environment occur through anthropogenic activities and natural incidents such as forest fires and volcanic eruptions. These pollutants can be transferred spontaneously by wind and/or rain into various ecosystems (Martínez-Lladó et al., 2007; Vergnoux et al., 2011). Persistence of PAHs in nature is often caused by their association with organic matter and nonaqueous-phase liquids (NAPLs), such as light or crude oil, creosote, coal tar and soot-like materials (Tejeda-Agredano et al., 2011; 2014; Ortega-Calvo et al., 2013). Different exposure regimes can be caused by exchange between these solid and liquid phases. This exchange relies on the physicochemical properties of PAHs, such as low water solubility and high hydrophobicity, and can have a profound impact on their bioavailability (Schwarzenbach et al., 2003; Mackay et al., 2006). The persistence of PAHs through sorption to inorganic solid surfaces, organic matter or NAPLs is therefore the major cause of reduced bioavailability for microbial degradation. Because bioavailability limits the effectiveness of PAH-degrading activity in bioremediation, some applications to enhance bioavailability, with a focus on their environmental risks, have been proposed (Ortega-Calvo et al., 2013). Among all applications, the mycelial networks of oomycete and fungi are an ecological application that could enhance the bioavailability of PAHs. However, mycelial networks are applicable only for increasing the bioavailability of flagellated PAH-degrading bacteria, e.g., *Achromobacter* sp., *Sphingomonas* sp. and *Pseudomonas* sp. (Kohlmeier et al., 2005; Wick et al., 2007; Furuno et al., 2010). Non-flagellated PAH-degrading bacteria, such as *Mycobacterium*

sp., have not been included in these ecological applications (Kohlmeier et al., 2005), which is problematic because they are found abundantly within PAH-polluted sites (Uyttebroek et al., 2006).

As the development and subsistence of zoospores within polluted environments and bioremediation scenarios are poorly understood, the ecological impacts of zoospores may provide additional knowledge for further improvements to innovative bioremediation technology. With this hypothesis, we assessed zoospore development of a rhizosphere oomycete, *Pythium aphanidermatum*, in different PAH-polluted environments. Two common PAH-degrading bacteria (*Mycobacterium gilvum* VM552 and *Pseudomonas putida* G7) were also used; these bacteria exhibit differences in their physiology and PAH-degrading capabilities. The development of zoospores was evaluated at three general stages of their life cycle: formation, taxis and settlement. The PAH-polluted environments were constructed with different levels of bioavailability, as determined by the different exposure regimes of PAHs in combination with chemical effectors. The term "chemical effectors" refers to a set of environmental chemicals that may have a profound influence on zoospore development and/or environmental relevance in PAH pollution and bioremediation scenarios. The possible applications of zoospores at each stage of their development to the improvement of bioremediation technology are discussed in this article.

## 2. Materials and Methods

### *2.1 Zoospore-producing organism and formation of zoospores*

The primary stock of the oomycete *Py. aphanidermatum* originated from the culture collection of Aberdeen Oomycete Laboratory, University of Aberdeen, United Kingdom. It was grown in diluted V8 juice (DV8) agar [4% (v/v) filtered Campbell's

V8 juice; 20 g agar powder (Panreac, Barcelona, Spain); 1 L distilled water] at 25 ºC. To form zoospores, 10 pieces (1 $cm^2$) of a 4-day-old hyphal mat growing on the agar were soaked with 10 mL of sterilized lake water (Embalse Torre del Águila, Seville, Spain) in a 50-mL Erlenmeyer flask. Zoospores were released after incubation at 25 ºC for 5-6 h. The zoospore density obtained by this process was $10^4$ zoospores $mL^{-1}$ (quantified in BLAUBRAND® counting chambers, BRAND GMBH + CO KG, Wertheim, Germany).

### *2.2 PAH-degrading bacteria*

The bacterial strains used in this study were *M. gilvum* VM552, which was isolated from PAH-polluted soil and is able to use phenanthrene, naphthalene, fluoranthene, pyrene and anthracene as sources of carbon and energy, and the motile naphthalene-degrading *P. putida* G7. *M. gilvum* VM552 was supplied by D. Springael (Catholic University of Leuven, Leuven, Belgium), whereas *P. putida* G7 was supplied by C.S. Harwood (University of Washington, USA). These bacteria were maintained in mineral salt media supplemented with phenanthrene for *M. gilvum* VM552 (Tejeda-Agredano et al., 2011) and naphthalene for *P. putida* G7 (Jimenez-Sanchez et al., 2012); the two PAHs served as the sole carbon and energy sources. For the purposes of tests with zoospores, the bacteria were routinely grown in tryptic soy broth (TSB) (Sigma-Aldrich, Germany) through shaking incubation at 150 rpm and 30 ºC for 4 days. Bacterial cells were harvested by centrifugation at 4303 × *g* for 5 min and then washed twice and re-suspended with sterilized lake water. The initial cell density of bacteria was adjusted to an optical density ($OD_{600\ nm}$) of 1.5. This OD corresponded to $10^{10}$ and $10^8$ colony-forming units (CFU) $mL^{-1}$ for *P. putida* G7 and *M. gilvum* VM552, respectively.

### *2.3 Antagonism tests and the influence of bacteria on zoospore formation*

The possible antagonistic effects between *Py*. *aphanidermatum* and PAH-degrading bacteria were studied within two different habitats, including a solid surface of agar media and a liquid phase of sterilized lake water. To determine the interaction during surface growth on solid media, an antagonism test was performed using a dual culture technique (Fokkema, 1978). Two different media [DV8 agar, and tryptic soy agar (TSA) (Sigma-Aldrich, Germany)] were used. These two media vary in terms of their compositions and the concentrations of total carbon that are available for growth. The estimated total carbon in TSA was 2% (w/v) and 0.2% (w/v) in DV8 agar; the composition and nutritional information about each medium was used to perform the estimation. First, the bacterial inoculum was streaked at ~2 cm from the edge of the agar plate, which was then incubated at 30 ºC until visible bacterial biomass developed. Then, an agar plug (Ø = 0.5 cm) of the oomycete (previously grown on DV8 agar at 25 ºC for 4 days) was placed opposite to the bacterial growth. The dual culture plates were incubated at 25 ºC and observed every day until the oomycete mycelia reached over the bacterial growth (~1 week). All tests were performed in triplicate.

To determine the interaction within an aqueous habitat, we performed a test for zoospore formation and used the number of zoospores produced as an indicator of the influence of the bacteria. A set of different bacterial cell densities was prepared and introduced into the zoospore formation system. The bacteria were grown for 4 days under the conditions described previously. Their initial cell density was adjusted to an $OD_{600\ nm}$ of 1.5, which was serially diluted 10-fold using sterilized lake water. The number of zoospores produced with dilutions of bacterial cells was counted and compared with the control without bacterial cells. The count was performed twice after

4 and 6 h of incubation. All tests were performed at least in triplicate. The highest cell density of each bacterium that did not exhibit a negative influence on zoospore formation was selected as the optimal cell density for further co-existence experiments.

### *2.4 Chemical effectors and preparations*

A set of chemical effectors was selected on the basis of their relevance for zoospore development, the environmental fate of PAH pollution and/or bioremediation scenarios. The chemical effectors were composed of organic solutions, polar solvents and non-polar solvents. The organic solutions included sterilized lake water, a number of root exudates and humic acids. The sterilized lake water was used for the production of zoospores and as a media solution for preparing the PAH-saturated solutions, which were used further for evaluating their effects on zoospore formation and taxis. Three root exudates of the representative plants applied in bioremediation, including *Helianthus*, *Festuca* and *Lolium*, were produced *in vitro* (see Supplementary Method S1 for the production protocol). Chemical properties of the root exudates are reported in Supplementary Table S1 and Fig. S1. Root exudates saturated with PAHs were also prepared for tests of their effects on zoospore taxis. Humic acids were collected from a soil sample taken from the Doñana National Park in Huelva, Spain. A solution of humic acids was prepared in 1 M NaOH, with a final concentration of 0.1% (w/v), and the pH was adjusted to 6 using HCl (Tejeda-Agredano et al., 2014). This solution of humic acids was also saturated with PAHs to test its effect on zoospore taxis.

The polar solvents (acetone and absolute ethanol) were purchased from Panreac, Barcelona, Spain. These two solvents are often used for extraction of organic pollutants that are found in nature, which increases the availability of pollutants for further treatments. In addition, their derivatives are also found as a component of root exudates,

and ethanol is a known attractant for diverse eukaryotic zoospores (Cameron and Carlile, 1978; Fan et al., 2002). These two solvents were selected to create the maximum exposure regime of PAHs dissolved in the aqueous phase. Co-exposure of the two solvents with PAHs was used for testing their effects on zoospore taxis. The non-polar solvents [hexadecane (HD) and 2,2,4,4,6,8,8-heptamethylnonane (HMN)] were purchased from Sigma-Aldrich, Germany. These two solvents are often found in environments that are polluted with oil, which acts as a reservoir for other hydrophobic pollutants, including PAHs. The two solvents were selected to create the lowest PAH exposure regime, thereby serving as an example of the very low bioavailability of PAHs found in polluted environments. Co-exposure of the two solvents with PAHs was used for testing their effects on zoospore settlement. All PAHs (naphthalene, fluorene, phenanthrene, fluoranthene, pyrene and anthracene) used in this study were supplied in crystal form and were purchased from Sigma-Aldrich, Germany.

### *2.5 Determination of different PAH exposure regimes*

Different PAH exposure regimes were created to evaluate the influences of PAHs on zoospore development within aqueous microenvironments. All PAHs were dissolved in pure dichloromethane (Sigma-Aldrich, Germany) and allowed to re-crystallize after complete volatilization of the solvent at ambient temperature for 12 h before use. The previously mentioned chemical effectors that contained PAHs were prepared by adding an excess amount of each PAH (100 mg $L^{-1}$) to them and maintaining them for 15 days to allow the PAHs to dissolve and/or saturate (Ortega-Calvo et al., 2003). The exposure regimes of PAHs were defined in terms of the free fractions of PAHs found in the aqueous phase after being directly exposed or co-exposed with chemical effectors to the aqueous microenvironments created in all tests performed in this study. The exposure

regimes were estimated using the exposure concentration ($C_{exp}$) of each PAH. At high exposure regimes of PAHs dissolved in chemical effectors made by polar solvents or in organic solutions, the $C_{exp}$ values of the PAHs were estimated based on their water solubility (Table 1). The chemical effectors made by polar solvents caused complete dissolution of PAHs, which created the maximum $C_{exp}$ values of the PAHs when they were co-exposed to the aqueous microenvironments. For the chemical effectors made by organic solutions that contained dissolved organic carbon (DOC), the $C_{exp}$ values of the PAHs were the result of their dissolution and sorption to DOC at equilibrium (after 15 days of saturation) (Schwarzenbach et al., 2003). These $C_{exp}$ values were estimated using equations (i) and (ii), and the results are presented in Tables 1 and 2.

$$f_w = \frac{1}{1 + [\mathrm{DOC}] \cdot K_{oc}} \qquad \text{(i)}$$

In equation (i), $f_w$ is the fraction of PAH dissolved freely in the aqueous phase at equilibrium, [DOC] is the concentration of DOC [in kg $L^{-1}$ of total organic carbon (TOC) in the solution presented in Table 2], and $K_{oc}$ (in L $kg^{-1}$) is the organic-carbon-normalized sorption coefficient of the considered PAH (Table 1). Once equilibrium was achieved after 15 days of saturation, $f_w$ corresponded to the aqueous solubility ($S_w$) of each PAH. Therefore, the $C_{exp}$ value for each organic solution was estimated using equation (ii):

$$C_{exp} = \frac{S_w}{f_w} \qquad \text{(ii)}$$

At low exposure regimes of PAHs dissolved in chemical effectors made by non-polar solvents, PAHs also dissolved completely, but these chemical effectors exhibited a phase differentiation with respect to the aqueous microenvironments. The differentiated phase of such chemical effectors caused the lowest $C_{exp}$ values of the PAHs when they were co-exposed to the aqueous microenvironments. Hence, the $C_{exp}$ values of the PAHs were estimated with the octanol-water partitioning coefficient ($K_{ow}$) listed in Table 1 using equation (iii), in which [PAH] is the concentration of each PAH added in these chemical effectors. The results of this estimation are presented in Table 2.

$$C_{exp} = \frac{[PAH]}{K_{ow}} \qquad \text{(iii)}$$

***2.6 Observations of zoospore development***

The number of zoospores formed by the oomycete was used as an indicator for assessment of the toxicity caused by individual PAHs in the absence and presence of PAH-degrading bacteria. The influence of PAHs on zoospore formation was assessed using all 6 of the PAHs (Table 1). The assessment was performed by quantifying the number of zoospores produced after 8-10 h of incubation in PAH-containing sterilized lake water compared with the PAH-free controls. For assessment in the presence of PAH-degrading bacteria, the optimal cell density of bacteria, which was described previously, was employed to quantify the zoospore formation. The PAHs that exhibited the strongest effects on zoospore formation were selected based on a statistical comparison and used to further test the influence of PAHs on zoospore taxis and settlement. These two behaviors of zoospores were analyzed in both the presence and absence of PAH-degrading bacteria.

The tactic responses of zoospores to the different chemical effectors listed in Table 2 (except for the non-polar solvents) were tested using a modified chemical-in-capillary method (Ortega-Calvo et al., 2003). The modification was the use of 50-µL capillary tubes (Microcaps®, Drummond, Broomall, PA, USA) as the arms of a chamber for creating an aqueous microenvironment by adding microbial suspension (Supplementary Fig. S2a). The chamber was inserted with 1-µL capillary tubes filled with the chemical effectors (Microcaps®, Drummond, Broomall, PA, USA). The experimental setups were left at 25 ºC for 0.5-1 h to allow the tactic responses and encystment of zoospores to develop. The tactic responses were quantified based on the number of zoospore cysts inside the capillary tubes. The levels of tactic responses were classified in accordance to appropriate statistical comparisons. The influences of the PAHs on the tactic responses of zoospores to the chemical effectors that contained PAHs were tested. The influence of PAH-degrading bacteria on the tactic responses of zoospores was tested using microbial suspensions of zoospores and each bacterium. The highest cell density of each bacterium that did not exhibit a negative influence on zoospore formation was used for preparing the microbial suspension. All tests were performed with at least four replications.

For observation of zoospore settlement, non-polar solvents (HD and HMN) either containing PAHs or not containing PAHs were used as test chemical effectors. Each of them was initially added into the same chamber mentioned above, followed by addition of microbial suspensions. Both chemical effectors formed a differentiated phase with respect to the water, and the interface constituted a substratum for zoospore settlement (Supplementary Fig. S2b). All of the developmental stages of the zoospores within the aqueous microenvironments were visualized using a phase-contrast Axioskop 2 Carl Zeiss microscope (Jena, Germany) connected to a Sony ExwaveHAD color video

camera (Tokyo, Japan), using either normal light or fluorescence mode. The fluorescence micrograph was taken after directly staining the chamber with 0.02% (w/v) acridine orange (Sigma-Aldrich, Germany). Representative micrographs were taken from the video records using the snapshot tool in Windows Movie Maker run on the Microsoft Windows XP operating system.

### *2.7 Statistical comparisons*

The comparisons of multiple means with standard deviations (SDs) among treatments were performed using the SPSS version 16.0 (SPSS, Chicago IL, USA) software package with appropriate analysis of variance (ANOVA) at different significance (*P*) levels (selected at $P = 0.05$ or lower). The *F*-distribution values, degrees of freedom and significance values are presented elsewhere in this article.

## 3. Results

### *3.1 Antagonistic effects among oomycetes and PAH-degrading bacteria*

The antagonistic interactions of *Py*. *aphanidermatum* and different PAH-degrading bacteria were evaluated based on their surface growth and growth in aqueous habitats. Based on the surface growth, no antagonistic effect was observed either on TSA (Fig. 1a, d) or DV8 agar (Fig. 1b, e). A dense growth of oomycete mycelium was observed over the *M*. *gilvum* VM552 biomass that developed on DV8 agar (Fig. 1b). Interestingly, *P*. *putida* G7 moved out from the inoculated area along the growing mycelia of the oomycete on TSA (Fig. 1d). When using repeated-measures ANOVAs with a Greenhouse-Geisser correction, the mean numbers of zoospores produced by the oomycete differed statistically between two continuous incubation periods (4 and 6 h) ($F_{(1, 23)} = 21.047$, $P < 0.0005$ (Fig. 1c) and $F_{(1, 23)} = 33.625$, $P < 0.0005$ (Fig. 1f)). *Post*

*hoc* tests using the Bonferroni correction revealed that the zoospore numbers increased significantly from 4 to 6 h of incubation ($P < 0.0005$) (Fig. 1c, f). With separate one-way ANOVAs, the number of zoospores decreased significantly only in the presence of the highest bacterial cell density tested with *M. gilvum* VM552 after co-incubation for 6 h ($F_{(5, 18)} = 11.266$, $P < 0.0005$) (Fig. 1c), and with *P. putida* G7 after co-incubation for 4 h ($F_{(5, 18)} = 18.185$, $P < 0.0005$) and 6 h ($F_{(5, 18)} = 14.985$, $P < 0.0005$) (Fig. 1f).

### *3.2 Zoospore development within PAH-polluted environments and bioremediation scenarios*

#### *3.2.1 Zoospore formation*

The primary stage of zoospore development is the formation of zoospores. Therefore, the number of zoospores produced by *Py. aphanidermatum* was used for evaluating the effects of different PAHs and/or PAH-degrading bacteria. The toxic influences of the PAHs on zoospore formation were different for each PAH (Fig. 2). A progression between toxicity and the estimated $C_{exp}$ value of the PAHs was observed. The $C_{exp}$ values of these PAHs are presented in Table 1. The relative toxicity of the PAHs exhibited the following sequence: naphthalene (91.67 ± 3.21%, mean reduction of zoospore formation ± SD) ≥ phenanthrene (84.72 ± 2.78%) = fluorene (73.61 ± 6.99%) ≥ fluoranthene (66.67 ± 4.54%) > pyrene (43.06 ± 18.36%) = anthracene (20.83 ± 15.96%). The sequence was arranged based on a one-way ANOVA of the mean numbers of zoospores in the absence of PAH-degrading bacteria ($F_{(6, 22)} = 49.806$, $P < 0.0005$). Based on this sequence, the PAHs with highest toxicity (naphthalene and phenanthrene) were selected for further tests in the subsequent stages of zoospore development (taxis and settlement).

The influences of PAH-degrading bacteria on zoospore formation were observed and explained with statistical analysis, using separate one-way ANOVAs. In the absence of PAHs, neither *M. gilvum* VM552 nor *P. putida* G7 cells influenced zoospore formation ($F_{(2,\ 10)}$ = 3.520, $P$ = 0.07). We also observed that both PAH-degrading bacteria diminished the toxic influence of all PAHs. However, this suppression was found at different levels; whereas *M. gilvum* VM552 was a greater detoxifier in phenanthrene solutions ($F_{(2,\ 9)}$ = 132.346, $P$ < 0.0005), *P. putida* G7 was more efficient in naphthalene solutions ($F_{(2,\ 9)}$ = 51.066, $P$ < 0.0005). Both PAH-degrading bacteria exhibited equal levels of toxic suppression with pyrene ($F_{(2,\ 9)}$ = 11.466, $P$ = 0.003), anthracene ($F_{(2,\ 9)}$ = 27.214, $P$ < 0.0005), fluorene ($F_{(2,\ 9)}$ = 19.840, $P$ = 0.001) and fluoranthene ($F_{(2,\ 9)}$ = 22.234, $P$ < 0.0005).

### *3.2.2 Zoospore taxis*

A consequent stage of zoospore development after formation is the swimming phase, in which the direction of swimming zoospores is often regulated by different chemical effectors that are found in nature. In this developmental stage of zoospores, their tactic responses to a set of chemical effectors that might contain PAHs were evaluated in both the presence and absence of PAH-degrading bacteria (Fig. 3). A three-way ANOVA of the results indicated statistically significant interactions among PAH-degrading bacteria, chemical effectors and PAHs in the tactic responses of zoospores ($F_{(24,\ 334)}$ = 17.182, $P$ < 0.0005). Through separate two-way ANOVAs (one for each bacterium and one for the absence of bacteria), we also detected statistically significant interactions between PAHs and chemical effectors that affected the tactic responses of zoospores either in the absence ($F_{(12,\ 202)}$ = 47.889, $P$ < 0.0005) or presence of *M. gilvum* VM552 ($F_{(12,\ 68)}$ = 9.591, $P$ < 0.0005) or *P. putida* G7 ($F_{(12,\ 64)}$ = 5.016, $P$ < 0.0005).

With these statistically significant interactions, we then tested whether PAHs and/or chemical effectors had an effect on the tactic responses of zoospores using simple main-effects tests.

In the absence of PAH-degrading bacteria, significant tactic responses of zoospores to polar solvents (acetone and ethanol) and *Festuca* root exudates ($F_{(6, 202)}$ = 165.157, $P < 0.0005$) were observed. Significant responses of zoospores to *Helianthus* and *Festuca* root exudates were also observed when they were co-exposed with naphthalene ($F_{(6, 202)}$ = 53.001, $P < 0.0005$) or phenanthrene ($F_{(6, 202)}$ = 53.705, $P < 0.0005$). The highest tactic response of zoospores to *Festuca* root exudates ($F_{(6, 68)}$ = 118.423, $P < 0.0005$) was observed in the presence of *M. gilvum* VM552, and this response was similar to the response when the root exudates were co-exposed with naphthalene ($F_{(6, 68)}$ = 272.824, $P < 0.0005$) or phenanthrene ($F_{(6, 68)}$ = 148.816, $P < 0.0005$). *M. gilvum* VM552 slightly enhanced the tactic responses of zoospores to *Helianthus* and *Festuca* root exudates that contained naphthalene, together with lake water, humic acids and *Festuca* root exudates that contained phenanthrene. Significant tactic responses of zoospores to *Helianthus* and *Festuca* root exudates ($F_{(6, 64)}$ = 64.177, $P < 0.0005$) were observed in the presence of *P. putida* G7. Significant responses to such root exudates were also observed when they were co-exposed with naphthalene ($F_{(6, 64)}$ = 47.968, $P < 0.0005$) or phenanthrene ($F_{(6, 64)}$ = 29.929, $P < 0.0005$). *P. putida* G7 slightly enhanced the tactic responses of zoospores to ethanol, humic acids and lake water that contained naphthalene and to humic acids that contained phenanthrene. The highest enhancement by *P. putida* G7 was found in the tactic response of zoospores to phenanthrene-containing acetone. However, the bacterium slightly reduced the tactic response of zoospores to *Helianthus* root exudates that contained phenanthrene. These

interpretations that were derived from simple main effects tests were confirmed with separate one-way ANOVAs.

Four levels (strong, medium, weak and least response) were assigned to classify the tactic responses of zoospores using separate one-way ANOVAs at the same significance level ($P = 0.01$) (Fig. 3). The statistical comparisons were performed across all tests of responses of zoospore taxis to chemical effectors that contained naphthalene ($F_{(20, 107)} = 136.698$, $P < 0.0005$) or phenanthrene ($F_{(20, 110)} = 100.652$, $P < 0.0005$) and to the control without PAHs ($F_{(20, 116)} = 56.541$, $P < 0.0005$). Tactic responses of zoospores that were similar to that observed in the control also occurred for most chemical effectors that contained PAHs, except for the chemical effectors that were made using polar solvents. A strong tactic response of zoospores to *Helianthus* root exudates was found only in the presence of *P. putida* G7 (Supplementary Vid. S1). In the control that did not contain PAH-degrading bacteria, zoospores exhibited weak-to-medium tactic responses to polar solvents and *Festuca* root exudates. However, zoospore cysts germinated only after exhibiting tactic responses to the root exudates. In addition, the tactic responses of zoospores to polar solvents were diminished when these chemical effectors were co-exposed with PAHs. Interestingly, the presence of PAH-degrading bacteria in the control also diminished the tactic responses of zoospores to polar solvents but not the responses to *Festuca* root exudates.

### *3.2.3 Zoospore settlement*

A typical developmental stage after the swimming phase of zoospores is settlement, in which they release their flagella and become cysts that may then attach to wet surfaces and germinate their germ tubes for mycelial growth. Chemical effectors (non-polar solvents) that created interfaces with water were used for observation of

zoospore settlement. We found that zoospores settled preferentially at the interfaces between water and PAH-containing or PAH-free hexadecane (HD) (Fig. 4 and Supplementary Vid. S2). There was no qualitative difference among all replicate observations (experiments were performed at least in triplicate). Settlement could be clearly observed through the accumulative colonization of zoospores, followed by encystment and germination after 1 h of incubation. The presence of PAHs (naphthalene and phenanthrene) dissolved in both chemical effectors (HD and HMN) and PAH-degrading bacteria did not influence the settlement behavior of zoospores. This implies that zoospore settlement was still maintained at the HD-water interface, and no settlement was observed at the HMN-water interface (Fig. 4). Accumulation of PAH-degrading bacteria in the settlement areas of zoospores was also observed (Fig. 5a, b and Supplementary Vid. S3). Side-view observations (Fig. 5c, d, and Supplementary Vid. S4) indicated that the germ tubes formed by zoospore cysts at the HD-water interface germinated and extended into the hydrophobic layer of HD.

## 4. Discussion

No antagonistic interaction between PAH-degrading bacteria and oomycetes or fungi has been reported (Kohlmeier et al., 2005; Wick et al., 2007; Furuno et al., 2012). These authors also revealed that the mycelia of oomycetes and fungi could facilitate the mobilization of PAH-degrading bacteria and/or PAHs, which later promoted the bioavailability and biodegradation of these pollutants. A similar mobilization of *P. putida* G7 through the mycelial surface of *Py. aphanidermatum* was observed in the antagonism tests performed in this study. However, this mobilization occurred only on a rich agar medium (TSA), which suggests that the bacterial mobilization might have been effectively enhanced through bacterial growth and the responses of chemotaxis to

dissolved nutrients along the oomycete mycelia. Additionally, dense mycelial growth of oomycete was found only on the biomass of *M. gilvum* VM552 that developed on limited agar medium (DV8 agar). Therefore, nutrient availability and exchange between oomycetes and bacteria were highly dependent on the excess amount of carbon and energy sources and/or selective bacterial species. Although the highest cell density of both PAH-degrading bacteria affected zoospore formation of the oomycete, such cell density was far greater than the natural density of cells in long-term PAH-polluted soil (Uyttebroek et al., 2006).

The toxic influence of PAHs on zoospore formation was dependent on either the $C_{exp}$ values, which exhibited a strong connection with the aqueous solubility of PAHs, or on the unique structure of the PAHs. Accordingly, naphthalene, because it had the highest water solubility of the PAHs used, produced the maximum toxic influence on zoospore formation. Interestingly, PAH-degrading bacteria could suppress the toxic influence of most of the PAHs tested, as evidenced by the significant enhancements in zoospore formation. The extent of suppression was different depending on the PAH and bacterial species. A restricted extent of toxic suppression was observed in the presence of naphthalene or phenanthrene, and significant differences were observed between the two bacterial strains. This result could be attributed to the dissimilar catabolic capabilities for PAH biodegradation because the toxicity of PAHs could be diminished by microbial degradation and transformation (Pagnout et al., 2006; Gandolfi et al., 2010). The toxicity of other PAHs was not significantly different when different bacterial species existed. This result suggests that the toxic suppression might occur through biosorption of PAHs to the microbial biomass. Indeed, either fungal or bacterial biomass can adsorb PAHs via partition mechanisms (Stringfellow and Alvarez-Cohen, 1999; Chen et al., 2010).

Zoospores possess at least a flagellum for translocation purposes, and their swimming period and distance vary depending on the species and surrounding microenvironment (Fan et al., 2002; Gleason and Lilje, 2009). Swimming can be maintained for up to two days in some fungal zoospores (Gleason and Lilje, 2009). The capacity of zoospore taxis constitutes an efficient dispersal tool for their homing, settlement, and colonization on target locations. To date, there are known tactic responses of rhizosphere zoospores to a set of attractants provided by plant roots (Cameron and Carlile, 1978; Fan et al., 2002; van West et al., 2002). We also found that the tactic responses of *Py. aphanidermatum* zoospores to *Festuca* root exudates were relatively strong and quite stable, without any influences by PAHs or PAH-degrading bacteria. Although some organic solvents, such as alcohols, are known to be attractants for several zoospores (Cameron and Carlile, 1978; Fan et al., 2002), the ethanol tested in this study was not an attractant for *Py. aphanidermatum* zoospores. Other studies have also reported that ethanol was not a chemotactic attractant for *Py. aphanidermatum* zoospores, but it can induce and enhance zoospore settlement and encystment (Jones et al., 1991; Donaldson and Deacon, 1993). Based on our observations, we suggest that the positive tactic responses of *Py. aphanidermatum* zoospores to such solvents were a result of induced settlement and encystment.

Interestingly, the response of zoospore taxis to *Helianthus* root exudates was enhanced when they were co-exposed with PAHs. The exact reason for this enhancement remains uncertain, but it might be related to a possible modification of the chemoattractants by their association with DOM. It is known that DOMs in the form of released microbial metabolites are one of the major factors that influence the tactic responses and swimming behaviors of marine microbes (Fenchel, 2002; Joint et al., 2002; Patel et al., 2003). *P. putida* G7 also caused the greatest enhancement of tactic

responses of zoospores to *Helianthus* root exudates, and this enhancement was not affected by PAHs. This bacterium-mediated enhancement could be caused by a combination of biological mechanisms (biosorption and biodegradation) and chemical association with DOM within root exudates (Haftka et al., 2008; Tejeda-Agredano et al., 2013). It is also possible that interactive motility of zoospores and bacteria might promote response of zoospore taxis to root exudates, which is similar to the biofilm formation of marine algae in the presence of bacterial population (Patel et al., 2003). Further studies of swimming behaviors and tactic responses of zoospores and bacteria are required for improving microbial accessibility in enhanced bioremediation. The highly attractive chemoresponses of zoospores to root exudates may expand the rhizosphere functions for phytoremediation of PAH-polluted environments.

PAHs are often found in the environment in association with hydrophobic materials, such as NAPLs, which causes a low bioavailability for microbial degradation and further limits the efficiency of bioremediation (Tejeda-Agredano et al., 2011; 2014; Ortega-Calvo et al., 2013). The developments of microbial community at the interfaces of pollutants with PAH-degrading capability are often required for effective biodegradation (Ortega-Calvo and Alexander, 1994; García-Junco et al, 2001; Tejeda-Agredano et al., 2011). Based on the settlement behavior of zoospores in our pollutant-water interface models, we observed that zoospores settled only at the HD-water interface. This settlement was not influenced by PAHs and PAH-degrading bacteria, but it seemed to occur through a selective chemoresponse to HD, whereas the methyl-branched chemical HMN was not recognized by *Py. aphanidermatum* zoospores. In addition, neither HD nor HMN was a carbon or energy source for growth of this zoospore-producing oomycete (Supplementary Fig. S3). Thus, the surface recognition was most likely related to the population community of zoospores themselves and/or

surface topography of such an interface (Greer et al., 2003; Schumacher et al., 2007; Heydt et al., 2012). Moreover, a recent study provided evidence that the specific settlement of oomycete zoospores, which is known as auto-aggregation, is a result of community-level interaction between zoospores, which is only regulated through chemotaxis and bioconvection mechanisms (Savory et al., 2014). After settlement, zoospores can germinate their germ tubes into the hydrophobic layer of HD. This may cause an increase of NAPL-water interfacial area and an enhancement of pollutant partitioning from such NAPL into the aqueous phase, which further increase the bioavailability of pollutants for bacterial degradation (Ortega-Calvo et al., 2013). We also found that co-existence with numerous cells of PAH-degrading bacteria in the settlement areas could potentially be an initiator of biofilm formation at the HD-water interface. The potential of biofilm as a strategy for promoting bioremediation of diverse pollutants is well documented (Singh et al., 2006). A number of studies also revealed that algal zoospores react chemotactically to quorum-sensing compounds produced by bacterial biofilms, which leads to a complex eukaryote-prokaryote community on wet surfaces within marine environments (Joint et al, 2002; Patel et al., 2003).

## 5. Conclusions

Our study suggests that the development of eukaryotic zoospores within PAH-polluted scenarios could yield a set of possible applications for improvement of bioremediation technology. The zoospore formation was susceptible to each PAH and was highly dependent on the bioavailability of each PAH. Quantification of zoospore number produced within PAH-polluted environments could provide an index of pollutant toxicity. The strong subsistence of zoospores and a set of positive interactions between zoospores and PAH-degrading bacteria with different exposures to PAHs were revealed in every developmental stage of zoospores. Possible enhancements of PAH

bioavailability through microbial dispersion by zoospore taxis and colonization at pollutant-water interfaces by zoospore settlement, germination, and further formation of mycelial networks were identified as further ecological applications of zoospores in bioremediation. The potent chemoresponses of zoospores are not only applicable for bioremediation; rather, they also suggest a number of possible approaches for controlling pathogenic and/or parasitic zoospores in nature. Based on this study, it is conceivable that modifications of PAH bioavailability using environmentally relevant chemical effectors such as humic acids and root exudates could either promote or diminish zoospore taxis and settlement. The bioavailability of PAHs should be considered when chemicals and/or biocides are applied in bioremediation and/or agriculture for proper use and management of eukaryotic zoospores.

## 6. Acknowledgements

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## Appendix A. Supplementary data

Supplementary data for this article can be found online at http://www.sciencedirect.com/science/article/pii/.

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**Table 1** Environmentally relevant properties of the PAHs used in this work

| PAHs | $S_w$ (mg $L^{-1}$)[a] | log $K_{ow}$[a] | log $K_{oc}$[b] | $C_{exp}$ (mg $L^{-1}$)[c] |
|---|---|---|---|---|
| Naphthalene | 31.3 | 3.37 | 2.98 | 31.3 |
| Fluorene | 1.9 | 4.18 | 3.78 | 2.0 |
| Phenanthrene | 1.1 | 4.57 | 4.16 | 1.3 |
| Fluoranthene | 0.26 | 5.22 | 4.80 | 0.41 |
| Pyrene | 0.13 | 5.18 | 4.76 | 0.20 |
| Anthracene | 0.045 | 4.54 | 4.13 | 0.050 |

[a)] The $S_w$ and log $K_{ow}$ values were taken from Mackay et al. (2006). [b)] The log $K_{oc}$ values were calculated using the equation $\log K_{oc} = 0.98 \cdot \log K_{ow} - 0.32$ (Schwarzenbach et al., 2003). [c)] The $C_{exp}$ value of each PAH dissolved in lake water, which contained 9 mg $L^{-1}$ of DOC, was calculated using equations (i) and (ii); see the text for details.

**Table 2** Exposure concentrations of naphthalene and phenanthrene in combination with different chemical effectors used for zoospore taxis and settlement assays

| Chemical effectors | TOC[a] (mg $L^{-1}$) | $C_{exp}$ (mg $L^{-1}$) | |
|---|---|---|---|
| | | Naphthalene | Phenanthrene |
| Non-polar solvents[b]: HD and HMN | NA | 0.043 | 0.003 |
| Polar solvents[c]: Acetone and ethanol | NA | 100 | 100 |
| Organic solutions[d]: | | | |
| Humic acids | 470[e] | 44.9 | 8.5 |
| *Helianthus* root exudates | 56.77 ± 1.97 | 32.7 | 2.0 |
| *Festuca* root exudates | 17.87 ± 0.35 | 31.5 | 1.3 |
| *Lolium* root exudates | 10.07 ± 4.05 | 31.3 | 1.3 |
| Lake water | 9.00 ± 0.12 | 31.3 | 1.3 |

Measurement of TOC was performed at least in duplicate, and the results are reported as the mean ± SD. [a)] The TOC was measured using a Shimadzu TOC-VCSH with ASI-V auto sampler after filtration through Whatman® No. 1 (pore size, Ø = 11 μm). [b)] The $C_{exp}$ values of PAHs that partitioned from these chemical effectors were calculated using equation (iii); see the text for details. [c)] Both PAHs were completely dissolved. [d)] The $C_{exp}$ values of PAHs dissolved in these chemical effectors were calculated using equations (i) and (ii); see the text for details. [e)] TOC concentration in 1 g $L^{-1}$ humic acids determined according to the elemental analysis by Lahlou et al. (2000).

**Figure legends**

**Fig. 1.** Antagonism tests for *Py. aphanidermatum* and PAH-degrading bacteria. The antagonistic effects of *M. gilvum* VM552 (a, b) and *P. putida* G7 (d, e) against mycelial growth of the oomycete on TSA (a, d) and DV8 agar (b, e) were tested using a dual culture assay. The arrows (d) indicate the mobilizing area of *P. putida* G7 along the growing mycelia of the oomycete. The influence on zoospore formation of the oomycete by *M. gilvum* VM552 (c) and *P. putida* G7 (f) was evaluated for two different incubation periods [4 (empty bar) and 6 h (filled bar)] using the zoospore production protocol; see the text for details. Asterisks denote significant differences of means compared with using separate one-way ANOVAs and Tukey's *post hoc* test.

**Fig. 2.** Influence of PAHs and PAH-degrading bacteria on zoospore formation. The number of zoospores formed after 8-10 h of incubation was used to determine the toxicity of PAHs in the absence of PAH-degrading bacteria (empty bar) and presence of either *M. gilvum* VM552 (light grey bar) or *P. putida* G7 (dark grey bar), compared with the control without PAHs. The graph shows the zoospore number, with error bars indicating the SDs, derived from at least four replicate results. In each PAH and control, values (means ± SDs) labeled with the same letter (or without a letter) are not significantly different (one-way ANOVAs and Tukey's *post hoc* test).

**Fig. 3.** Zoospore taxis in response to different chemical effectors that did not contain PAHs (control) or contained phenanthrene or naphthalene. Chemical effectors made by polar solvents and organic solutions were used in this evaluation. The $C_{exp}$ values of each PAH that was co-exposed with different chemical effectors are listed in Table 2.

Tactic responses of zoospores to different chemical effectors were evaluated in the absence (empty bar) and presence of PAH-degrading bacteria [*M. gilvum* VM552 (light grey bar) or *P. putida* G7 (dark grey bar)]. All figures show the means, with error bars indicating the SDs, derived from at least four replicate results. Values (means ± SDs) labeled with the same letter are not significantly different (one-way ANOVA and Tukey's *post hoc* test). The absence of a letter indicates less or weak tactic responses.

**Fig. 4.** Zoospore settlement in the absence and presence of PAH-degrading bacteria at the pollutant-water interfaces. The chemical effectors were prepared by PAH-containing or PAH-free HD and HMN. In the presence of PAH, 0.1% (w/v) of naphthalene or phenanthrene was used for the preparation. The arrows indicate zoospores that are randomly touching or settling on the interfaces of water and HMN or HMN containing PAHs. Bars = 50 μm.

**Fig. 5.** Co-settlement of *Py. apphanidermatum* zoospores with *M. gilvum* VM552 (a) or *P. putida* G7 (b) was observed at the HD-water interfaces. A fluorescence micrograph (a) that shows *M. gilvum* VM552 cells (arrows, green particles) that settled around and inside the germinated area (white circle) of zoospore cysts on the HD-water interface. The enlarged scale of the light micrograph (b) shows *P. putida* G7 cells (arrows) co-settled with germinated zoospore cysts at the HD-water interface. Side views of zoospore settlement at the HD-water interface (c) and HMN-water interface (d) were also examined using the chemical-in-capillary method; see the text for details. The zoospores encysted and germinated their germ tubes (arrows) only in the HD layer (c). All observations were performed at least in triplicate. Bars = 50 μm. All images were taken from Supplementary Vids. S2-S4.

**Fig. 1**

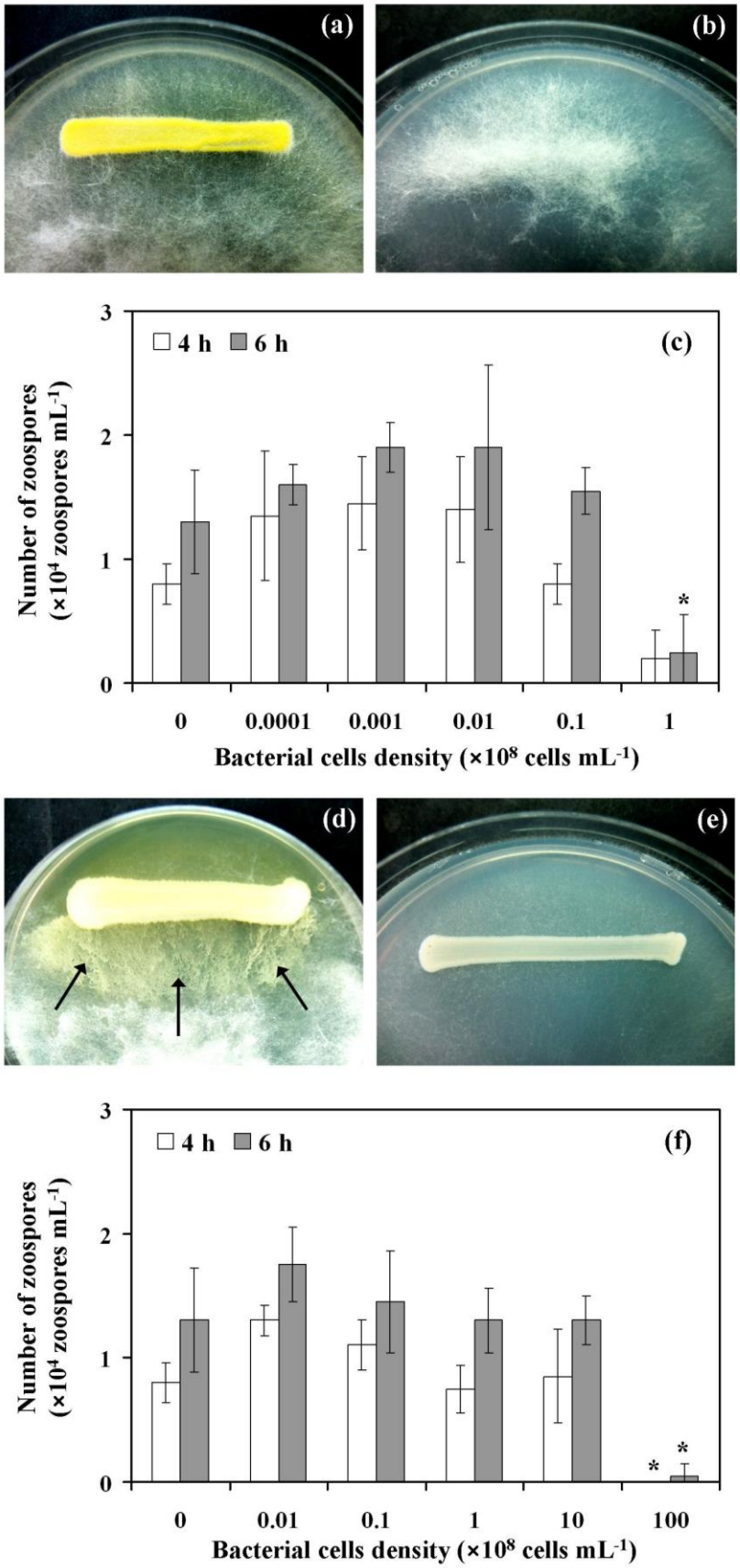
(a)
(b)
(c)
4 h
6 h
Number of zoospores (×10⁴ zoospores mL⁻¹)
0
0.0001
0.001
0.01
0.1
1
Bacterial cells density (×10⁸ cells mL⁻¹)
(d)
(e)
(f)
0
0.01
0.1
1
10
100

**Fig. 2**

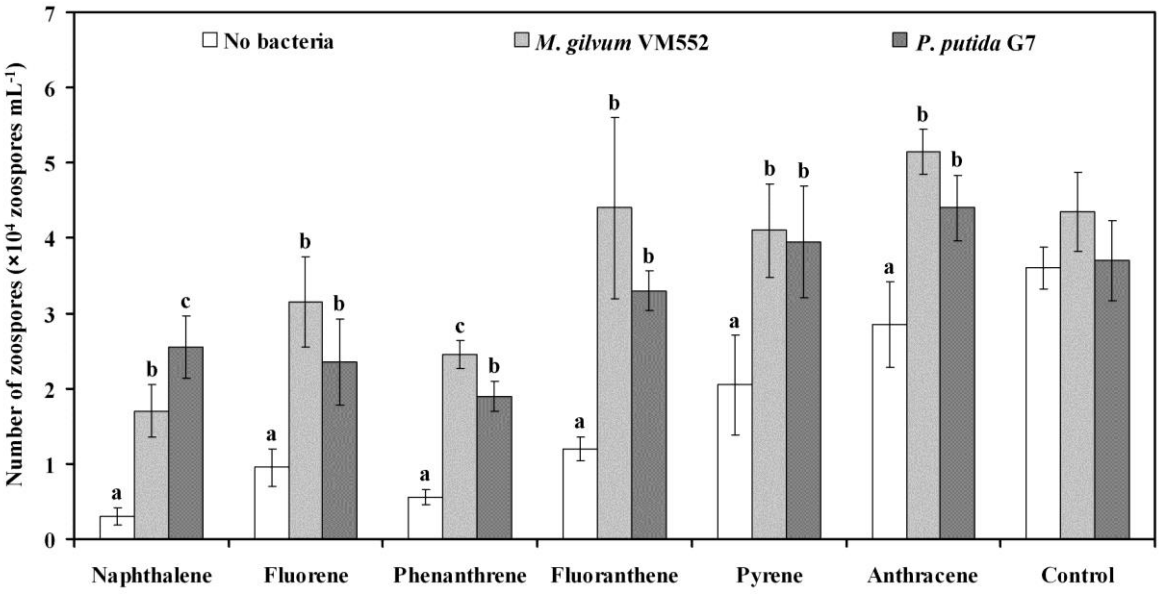
No bacteria
M. gilvum VM552
P. putida G7
Number of zoospores (×10⁴ zoospores mL⁻¹)
0
1
2
3
4
5
6
7
a
b
c
a
b
b
a
c
b
a
b
b
a
b
b
Naphthalene
Fluorene
Phenanthrene
Fluoranthene
Pyrene
Anthracene
Control

**Fig. 3**

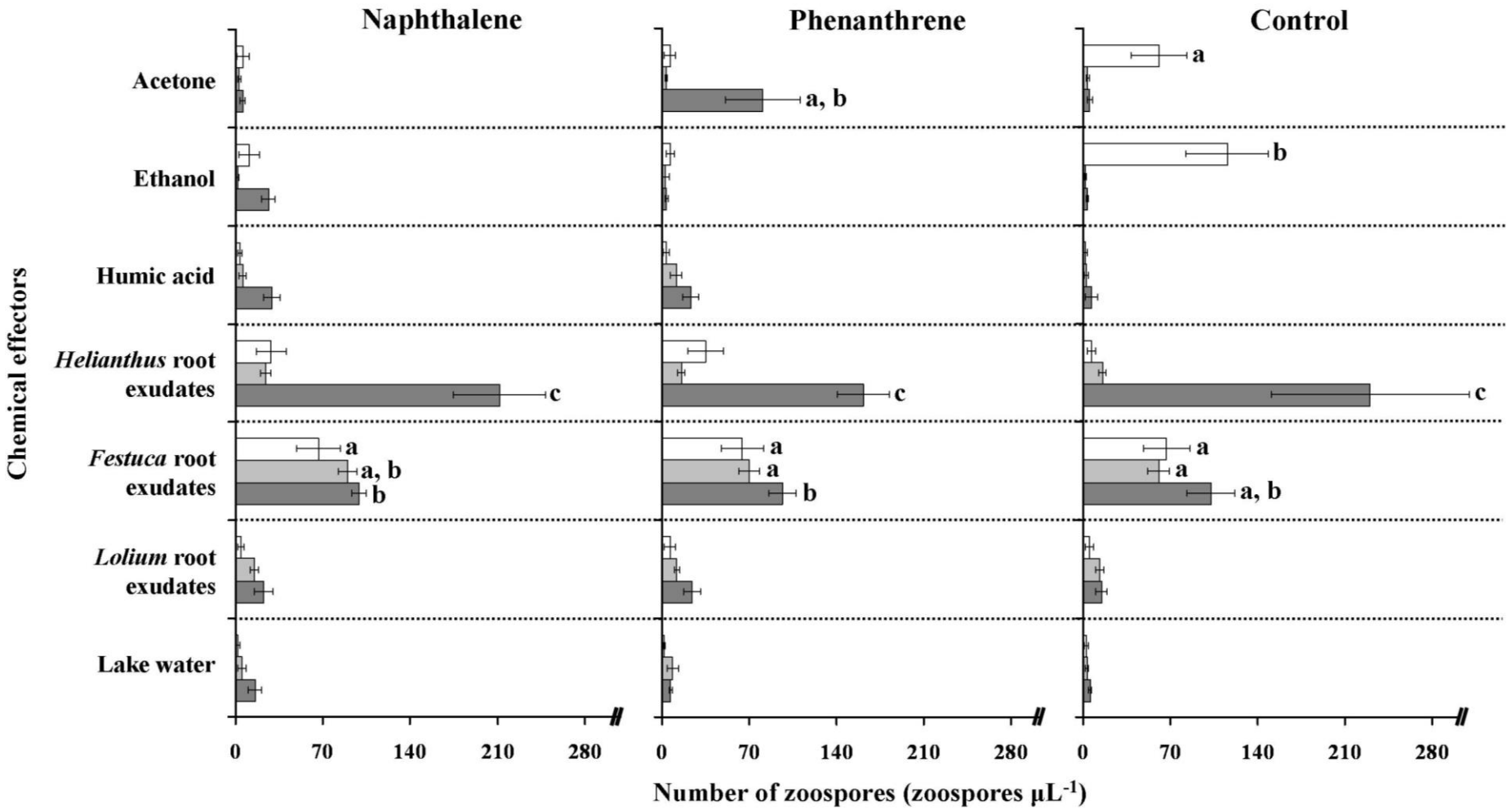
Naphthalene
Phenanthrene
Control
Chemical effectors
Acetone
Ethanol
Humic acid
Helianthus root exudates
Festuca root exudates
Lolium root exudates
Lake water
a, b
b
c
a
a, b
0
70
140
210
280
Number of zoospores (zoospores µL-1)

**Fig. 4**

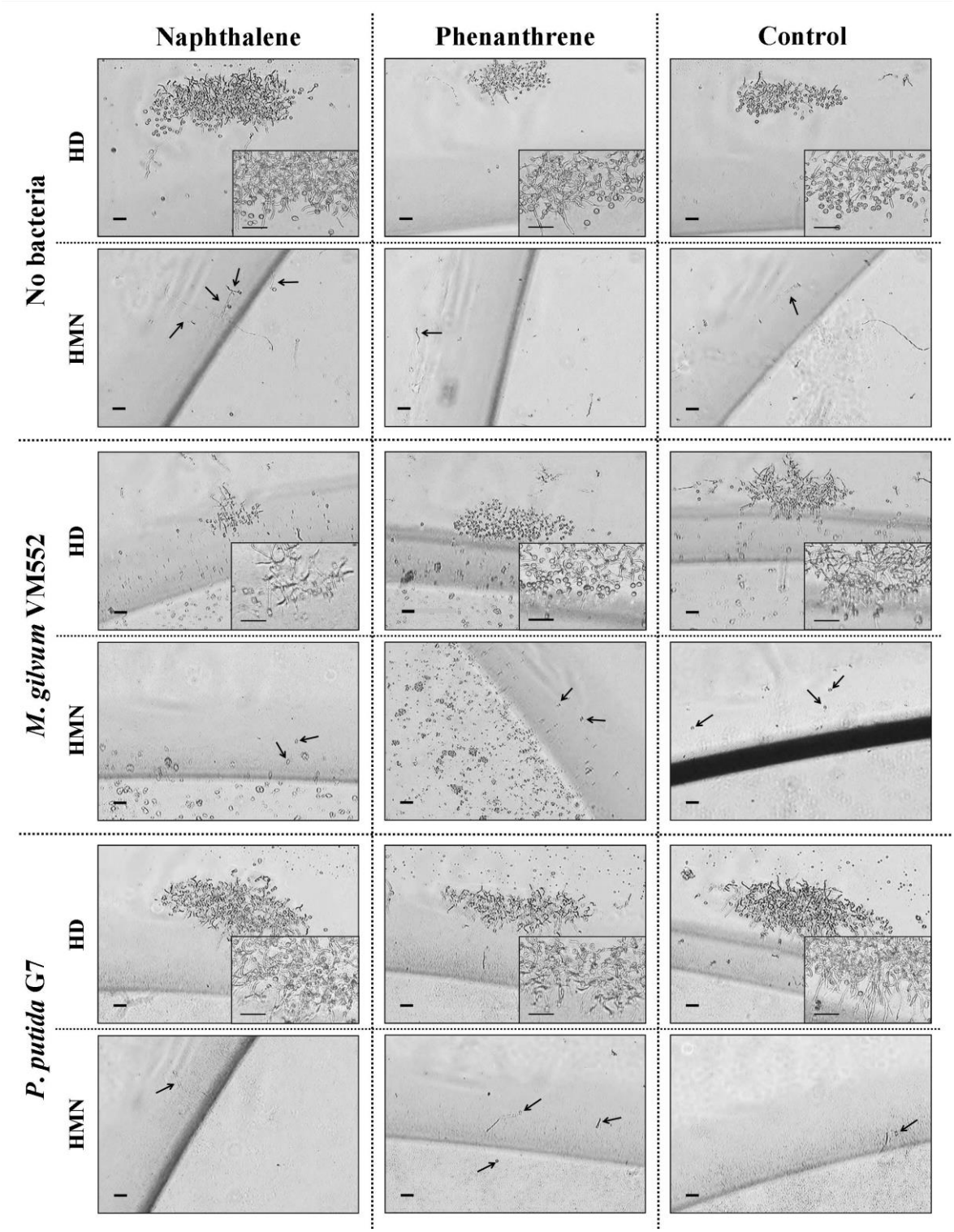
Naphthalene
Phenanthrene
Control
No bacteria
HD
HMN
M. gilvum VM552
HD
HMN
P. putida G7
HD
HMN

**Fig. 5**