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The Role Fungi and Yeast in Monitored Natural Attenuation

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ABSTRACT

Fungi and yeast have been characterized as important components in the bioremediation of organic contaminants in soil and water including polyaromatic hydrocarbons (PAHs); however, research into their ability to metabolize these compounds in extreme environments has been limited. In this work forty-three fungi and yeasts were isolated from a PAH-contaminated sludge waste lagoon in Poland. The lagoon was part of a monitored natural attenuation (MNA) study where natural reduction of PAHs and associated toxicity over time in non-disturbed areas of the sludge lagoon indicated MNA activity. The microorganisms were initially isolated on minimal medium containing naphthalene as the sole carbon and energy source. Fungal isolates were then maintained on MEA and identified based on microscopic examination and BIOLOG®. The analysis identified several of the fungal isolates as belonging to the genera *Penicillium*, *Paecilomyces*, *Aspergillus*, and *Eupenicillium*. Yeasts included *Candida parapsilosis* and *C. fluvialitis*. Further microbial characterization revealed that several isolates were capable of growing on acidified media of pH 4, 3, and 2.5. Over twenty percent of the fungi demonstrated growth as low as pH 2.5. Of the 43 isolates examined, 24 isolates exhibited growth at 5°C. Nine of the fungal isolates exhibiting growth at 5°C were then examined for metabolic activity using a respirometer testing metabolic activity at pH 3. Microcosm studies confirmed the growth of the fungi on PAH contaminated sediment as the sole carbon and energy source with elevated metabolic rates indicating evidence of MNA. Our findings suggest that many of the Poland fungal isolates may be of value in the bioremediation processes in acidic waste sites in northern climates typical of Northern Europe.

Keywords: Polyaromatic hydrocarbons, Monitored Natural Attenuation (MNA), Fungi, Yeast

INTRODUCTION

Monitored natural attenuation (MNA) as an environmental restoration option relies on natural biological and physical processes to contain the spread of contamination from a source (Brigmon et al., 2002). The biological components of MNA may include plants, bacteria, fungi, and yeasts. MNA is most appropriate when the concentrations of contaminants are reduced to regulatory limits before surface or groundwater impacts the environment or is collected by a monitoring well (Wilson et al., 1985). Rates of contamination removal over time by MNA can be estimated from microcosm studies and evidence of *in situ* contamination degradation (Wiedemeier et al., 1995). Natural physical chemical contaminant/soil/water interactions can also contribute to MNA by sorption, advection, dilution, volatilization, or oxidation (Aislabie et al., 2004).

This project focuses on MNA from the perspective of bioremediation of polycyclic aromatic hydrocarbons (PAHs) in cold low pH environments. PAHs are ubiquitous pollutants that can be carcinogenic as well as genotoxic in petroleum contaminated sites (Alexander & Alexander, 2000). The use of microorganisms and associated extracellular enzymes for environmental restoration of polluted sites is a promising and growing area of environmental biotechnology (Cerniglia and Sutherland, 2004). The involvement of microorganisms in the biodegradation of hydrocarbons contaminated waste sites has demonstrated that a consortia of fungi, bacteria, and yeasts are needed to mineralize these contaminants (Stapleton et al., 1998). For example, PAH-contaminated soil inoculated with *Phanerochaete ostreatus* or *P. chrysosporium*, demonstrated transformation of the PAH anthracene to the metabolite 9,10-antracendion (Andersson et al., 2000, Andersson and Henrysson, 1996). Other microorganisms are needed for further breakdown of the PAHs (Field et al., 1992; Andersson and Henrysson, 1996). In soil remediation, it has been shown that PAH degradation can be achieved under the right environmental conditions either by bioaugmentation, adding an inoculum, or with the indigenous soil microorganisms (Kim and Lee, 2007). However, an overall decrease in site/soil contaminant concentrations is not the sole criterion needed to effectively determine the effectiveness of a specific bioremediation approach, and consequently, a direct determination of the residual toxicity is required (Plaza et al., 2008). The biodegradation activity of

autochthonous fungi in PAH contaminated sites has been shown to contribute to significant decreases in soil toxicity (D'Annibale et al. 2006).

This work was in collaboration with a remediation project at the sulphuric acid-based petroleum refining at the Czechowice Oil Refinery (CZOR) in southern Poland (Plaza et al., 2003). In 1997, a PAH contaminated waste lagoon soil was engineered into a biopile with actively and passively aerated sections (Plaza et al, 2004). The project focus was on evaluation of novel environmental restoration technologies and research for effective bioremediation of heavily contaminated petroleum waste soils. The results confirmed the hydrocarbon biodegradation process by correlation of petroleum hydrocarbon concentrations, microbial respiration, toxicity assays, and microbial enzymes assays (Plaza et al., 2005). The evaluation examined bioremediation processes and contaminant removal rates of both easily biodegradable light hydrocarbons and more complex PAHs under active vs. passive remedial activities including MNA.

In previous microbiological studies associated with environmental restoration of CZOR, examination of acidophilic microorganisms and keratinolytic/keratinophilic fungi demonstrated the bioremediation potential of the indigenous microorganisms (Ulfig *et al.*, 2003). The microbial metabolism and fate of hydrocarbons including total petroleum hydrocarbons (TPHs), PAHs and straight-chain and branched alkanes in the natural environment are areas of intense concern since many of these compounds and their break-down products display toxic and carcinogenic properties (Bartha, 1984). Bacteria isolated from the sludge lagoon have been successfully applied for remediation at other PAH-contaminated sites (Berry et al, 2003.) The site currently exists as a green space within the refinery property. However, the biopile soil and leachate still contain residual and toxic hydrocarbon contaminants and, therefore, require further monitoring and study. The present project chiefly concerns MNA activity of indigenous fungi for the residual contamination in the Northern Europe environment.

Fungi are unique among microorganisms in that they secrete a variety of extracellular enzymes that can act as powerful catalysts in the biodegradation of PAHs (Gianfreda and Rao, 2004). The ability of fungi to transform a wide variety of hazardous chemicals has generated interest in using them in bioremediation. Studies have shown the white rot fungus *Phanerochaete sordida* was capable of degrading efficiently the three ring PAHs, but less efficiently the four-ring PAHs (Davis et al., 1993). Another fungus, *P. chrysosporium*, has also been shown to

degrade a number of aromatic hydrocarbons such as benzo alpha pyrene, phenanthrene, and pyrene (Kennedy et al., 1990). The white rot fungi *Ceriporiopsis subvermispora* and *Cyathus stercoreus* growth and lignolytic enzyme production been shown to be stimulated by certain aromatic compounds (Setheuraman, et al., 1998). Yeasts have also demonstrated the ability to degrade aromatic organic compounds through the identification of oxidation and ring cleavage products (Hammer et al., 1998).

The overall objective of this study was to identify MNA associated fungi and yeast obtained from this acidic site and to investigate isolated strains for bioremediation capacity of similarly cold climate PAH-contaminated areas. The isolates were tested for ability to grow on PAH containing sediments at decreasing levels of acidity. Fungal isolates were also classified using the BIOLOG® ™ system substrate level identification (Kim & Lee, 2007). The isolates were further characterized for ability to grow at cold (5°C) temperatures and metabolic activity at low pH (2.5) demonstrating MNA ability for the CZOR facility.

MATERIALS AND METHODS

Isolation and Culture of Fungal Isolates. The fungal isolates were originally obtained from a PAH-contaminated sludge from an untreated waste lagoon at the Czechowice Oil Refinery (CZOR) near the city of Czechowice-Dziedzice, in Southern Poland. For more than one hundred years the CZOR has been producing petroleum products for industrial and commercial applications. Many of the refinery's products are specialty items including semi-synthetic motor oils, hydraulic oils, high temperature lubricants and waxes. Catalytic cracking processes which generated acidic waste material were used to refine crude oil as this site. Both solid and liquid waste from the acid refinery process was historically disposed in lagoons on site. The current contaminated sludge in the lagoons is a partially characterized viscous semi-liquid hydrocarbon mixture (Plaza et al., 2003). Fifty grams of fresh lagoon sludge was initially suspended and mixed in 200 ml of phosphate buffered saline (PBS) for 1 week at 25°C to release microorganisms from the oily matrix and allow them to grow at operant conditions. After one week 0.1 ml was aseptically taken from the suspension and streaked onto noble agar (Difco) petri plates. The plates were then placed in a sealed stainless steel chamber supplied with naphthalene (Fisher Scientific) as a sole carbon and energy source at 25°C. After one week colonies that

developed were transferred and grown with the same medium and growth conditions. Each culture was reisolated and restreaked at least six times on this agar in the naphthalene supplied chamber to confirm growth. The isolates were maintained for continued growth and colony purification on MEA plates at 25°C. A Laser Scanning Confocal Microscope (LSCM) (Model 510, Carl Zeiss Inc, Thornwood, NY) was used to identify and characterize the microorganisms in the original PAH-contaminated sludge and isolates described here. Both Acridine Orange (AO) (Fisher) and Baclight (Molecular Probes) was used to stain the both the original sludge samples and isolates for microscopic examination.

Fungal and Yeast Growth at 5ºC and Acidic Conditions. Fungal and yeasts isolated using naphthalene as the sole carbon and energy source were next tested for growth at 5°C and then on acidified media. Each fungal isolate was restreaked on MEA slants and placed in a cold room at 5^{0}C. A duplicate set of inoculated cultures was kept at room temperature (25º C). Slants were checked for growth after 3 days. Uninoculated medium controls were incubated at both temperatures. Growth was determined by fungal colonies growing at the surface.

Fungi and yeast for pH testing were grown on 2% Oxoid Malt Extract Agar (MEA). After the medium was prepared an aliquot was taken and the pH was measured (initial pH = 6). The medium was adjusted to appropriate pH by titration with 85.8% lactic acid solution (Fisher Scientific). Media pHs included pH 4.0, 3.0, 2.5, 2.0, and 1.5. Due to solidification problems with acidified agar, all pH-altered medium used in growth studies was in liquid form. All fungal isolates were initially grown in pH 4 media at 25°C. Fungi that grew at pH 4 were then transferred to pH 3 media and allowed to incubate at 25°C between 72 to 96 hours. Those cultures that grew in pH 3 media were transferred to pH 2.5 and allowed to incubate at 25°C between 72 and 120 hours. Isolates that grew at pH 2.5 were transferred to media of pH 2 and 1.5 simultaneously and were monitored for viability. Controls included cultures grown at pH 7 and negative controls containing medium only.

BIOLOG™ Identification of Fungal Samples. The BIOLOG™ system (Hayward, CA) was used to identify and characterize the isolated fungi and yeast based on substrate utilization (Kim and Lee, 2007). Each pure filamentous fungal isolate was grown on a 2% Malt Extract Agar plate until enough conidia were present to prepare a suspension. The conidia were then swabbed

from the agar plate and suspended in sterile BIOLOG™ FF inoculating fluid. The suspension was adjusted to the acceptable turbidity range, as defined by the BIOLOG™ supplied turbidity standard and immediately pipetted into each well of ~~in~~ the 96-well BIOLOG™ FF MicroPlate (100 µl per well). After inoculation, the MicroPlate was incubated at 26°C for 24 to 96 h. Each MicroPlate was read at 24, 48, and 96 hours in a BIOLOG Microstation Reader which measured both color development and turbidity. The BIOLOG™ Reader results paired with macroscopic and microscopic morphology were used to provide an ID of each filamentous fungal culture. Each pure yeast isolate was grown overnight on a BIOLOG™ BUY agar plate. The cells were then swabbed from the agar plate and suspended in sterile 0.2u filtered deionized water. The suspension was adjusted to the acceptable turbidity range, as defined by the BIOLOG™ supplied turbidity standard and immediately pipetted into each well of a 96-well BIOLOG™ YT MicroPlate (100 µl per well). After inoculation, the MicroPlate was sealed and incubated at 26°C for 24 to 72 h. When a distinctive color pattern had formed in the microplate (time varied per culture), the MicroPlate was read in a BIOLOG Microstation Reader. The BIOLOG™ results paired with macroscopic and microscopic morphology were used to provide an ID of each yeast culture.

Respirometic analysis of fungal microcosms. Metabolic activity of fungal isolates in PAH contaminated soils was determined using a Respirometer (Columbus Instruments, Columbus, OH). Microcosms were made by adding fifty gm of fresh PAH-containing CZOR lagoon samples to 250 ml microcosm chambers of the respirometer. One gram wet weight of select fungal culture was collected by sterile pipette and weighed from a fresh over night culture and then added to each chamber. Protein concentrations of the inoculums were determined using the Coomassie Dry™ Protein Assay (Pierce, Rockford, IL). Constant temperature (25ºC) was maintained through the Respirometric analysis. The O_2 consumption and CO_2 production (ml/min) were measured simultaneously on-line each hour during a 24-hour time period. Each sample was measured in triplicate. Cumulative curves were plotted as O_2/CO_2 content values in the headspace versus time. Rates of O_2 uptake and CO_2 production were obtained by the slopes of the linear regression for the cumulative curves.

RESULTS

Initial microscopic analysis of the raw PAH-contaminated sludge samples, showed both filamentous fungal-like microbial structures (Figure 1) and yeast-like microorganisms (Figure 2) present. The selection process for the isolation method by repeated axenic culture using naphthalene as the sole carbon and energy source over a two month period resulted in 43 individual fungal and yeast isolates obtained from the Poland site.

Isolates cultured at 5°C took an average of 72 h for growth to be detected. The isolates were grown on non-acidic MEA medium. The temperature in the cold room was a replication of the seasonal environmental conditions that are typically found in the Polish CZOR. Of these 48 isolates examined, 28 exhibited visible growth at 5°C (Table 1).

A total of 34 isolates exhibited growth in pH 4 and pH 3 medium after 72 h. Only 16 of these 35 isolates also exhibited growth in the pH 2.5 medium. None of the isolates grew at pH 2 and pH 1.5. (Table 2). In this work pH of 2.5 may represent a cut-off of acid tolerance for the isolates observed to grow at this range, but since incremental growth studies using pH ranges below 2.5 were not conducted, it cannot be ruled out that these isolates could grow at pHs between 2.5 and 2.0. The genus and species of the isolates were classified using the BIOLOG™ system that uses pre-selected carbon sources to provide specific metabolic fingerprint. The BIOLOG™ identified only 13 of the 35 isolates (Table 2). These isolates belonged to the genera *Penicillium, Paecilomyces, Aspergillus, Candida*, and *Trichoderma*. This finding correlates well with studies that have shown that these genera are capable of degrading PAHs (D'Annibale et al., 2006).

Nine of the twenty-four isolates that exhibited growth at 5ºC were tested for metabolic activity using a respirometer. The samples were grown on acidified MEA medium (pH 3), which corresponds to the acidity range of the CZOR from which the fungal isolates were originally taken. All 9 isolates demonstrated the ability to consume oxygen and emit carbon dioxide during low pH respirometer testing (Table 3). The amount of CO_2 produced or O_2 consumed was measured as ul of gas emitted/ consumed per hour per gram (wet weight) of fungal isolate (Table 3). These findings suggest that the fungal isolates examined are able to maintain metabolic processes in an acidic environment.

DISCUSSION

MNA is a risk management option that relies on natural biological and physical processes to contain the spread of contamination from a source. The fact that microorganisms can metabolize PAHs that are toxic to other species for their growth and energy make them very useful (Johnsen et al., 2002). The biological components of MNA can include yeasts and fungi present in the soil as those tested here. While not all microorganisms can be readily isolated and cultured, the robustness of these strains to survive repeated isolation with naphthalene as the sole energy and carbon source demonstrates their bioremediation capacity. Microorganisms have been applied extensively for bioremediation of organic contaminants in soil and water including PAHs. PAHs are a class of recalcitrant organic compounds that makeup a large proportion of the CZOR refinery waste lagoon as previously described (Plaza et al.2006). Although the PAHs have been reduced in concentration due to environmental restoration activities, they remain a concern and need to be monitored. Because PAHs pose a threat to human health and compromise the integrity of the environment, the removal and/or attenuation is of high importance. However, the presence of heavy metal, alkaline or acidic pH, and temperature extremes greatly affect the rate of PAH degradation (Stapleton et al, 2000).

Fundamental questions that remain relevant to MNA include the site-specific rates of *in situ* microbial biodegradation, the relative contributions of fungi compared to bacteria; overall microbial contributions to nutrient cycling; microbial community adaptations to cold temperature, nutrient limitation, and resistance to desiccation, ultraviolet irradiation, influence of seasonal freeze-thaw cycles, and contaminant toxicity (Aislabie et al., 2004). Over time both moisture and sunlight can enhance PAH degradation including recalcitrant anthracene (Nunez et. al, 2009). While sunlight will of course only impact those materials in the top (e.g. 2 cm) of soil, landfarming practices of intermittent mixing contaminated soil and maintaining appropriate moisture levels over can achieve accelerated remediation rates by combining physical and biological degradation of these compounds (Brigmon et al., 2002).

It has been demonstrated that the specific stimulation of indigenous fungi is a promising method to mobilize PAH degrading bacteria and thereby improve *in situ* soil bioremediation (Wick et. al. 2007). This synergistic remedial action is importance as a number of bacteria, fungi, and yeast that can degrade PAHs have been identified (Cerniglia.2001). The fungal

isolates examined in this study were obtained from the waste lagoon of an oil refinery located in Czechowice, Poland. This waste lagoon contains a mixture of PAHs, low pH, and high amounts of heavy metals (Plaza et al., 2003). In addition, the region in which the oil refinery is located routinely experiences harsh, cold weather. Several of the strains identified here have been isolated from other contaminated sites and proven to degrade PAHs. For example, *P. simplicissium* and *T. harzianum* have been previously isolated and shown to degrade pyrene (Ravelet et al., 2000).

The lack of biodegradation of the PAHs in certain contaminated soils can be due to the PAHs being in a non-bioavailable form, for example, bound to soil particles in hydrophobic soil microenvironments, leaving them inaccessible to the soil microorganisms (Andersson et al., 2000). Nonetheless, this simple approach has been proven to explain the crucial impact of mass transfer on substrate (in this case PAHs) degradation and bacterial growth in terms of a flux-based approach. This PAH recalcitrance to microbial metabolism in soils can make it difficult to predict biodegradation or mass transfer paths, such as desorption or biosurfactant-solubilization, and develop a universal approach to predict the influence of mass transfer limitation on microbial growth (Wick et al, 2001). However, biosurfactant producing bacteria have been isolated and characterized from these same CZOR soils (Plaza et al., 2008), so further research on these microbial-soil-PAH interactions would be essential for development of effect contaminant removal models.

Although it has been shown in other work that indigenous microorganisms in aged petroleum-contaminated soil could degrade PAHs significantly, recent studies have shown that select introduced or bioaugmented microorganism have the capability to significantly enhance PAH biodegradation (Li et al., 2009). Fungi can produce key enzymes with high activity rates that have been found to degrade PAHs and actually solubilize coal (Faison et al., 1991). Application of laccase, an enzyme produced by many fungi species, has been found to efficiently decrease PAH genotoxicity with minimal impact on other microorganism, suggesting that laccase can be an efficient and safe remedial agent, especially where there is heavy contamination (Wu et al., 2008). As an alternative to adding microorganisms or other biological agents, the selective enrichment of indigenous microorganisms for PAH degradation, given the time and experimentation, may result in successful performance at a given site (Mohammed et

al., 2007). Whether the isolates described here would also grow and metabolize PAHs in warm climates under different conditions where the temperature may reach 40 ºC like other strains (Zinjarde and Pant 2002) remains to be tested. Many commercial bioremediation products are often utilized for petroleum related remediation without proper assessment of their effectiveness or suitability under local environmental conditions in relation to indigenous microorganisms, soil and/or water chemistry (Mohammed et al., 2007). These facts can help explain why bioremediation efforts are not always successful.

The recovery and maintenance of fungal isolates from this acidic waste lagoon allows speculation that some of these fungi may be useful in future bioremediation efforts of contaminated areas with acidic conditions (e.g. other refineries or mining sites). Isolates with high acid tolerance and metabolic rates would be most useful in such sites. In conclusion, our results demonstrated that 65% (28/43) of the purified fungal an yeast isolates could grow under cold (5ºC) conditions. These strains can potentially interact synergistically with other microorganisms that have similar activity under these same extreme conditions to attenuate contaminants (Stapleton et al., 1998; Gemmel & Knowles, 2000). Furthermore, all 9 isolates tested for metabolic activity at pH 3 yielded positive growth results. This work provides evidence of MNA activity for these isolates from the CZOR refinery. These findings suggest that some of these nine fungal isolates maybe useful in remediating acidic PAH-contaminated site that are exposed to similar extreme conditions.

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Table 1. Growth* of Fungal and Yeast Isolates at 5ºC After 72 h.

Isolate	Result	Isolate	Result
49-1a	-	PF-28	+
49-1c	+	PF-30B	+
52-1	+	PF-36	+
52-1b	-	PF-39	+
53-11A	-	PF-39	+
53-11B	-	PF-4	-
53-2B	-	PF-41A	-
53-2c	+	PF-46	+
53-3DA	-	PF-46A	+
53-6A	+	PF-46B	-
53-6c	-	PF-49	+
65-1	+	PF49B	-
PF-14B	+	PF-5	+
PF-2	-	PF-53	+
PF-21	-	PF-55	-
PF-21A	+	PF-9	-
PF-23C	+	PF-9C	-
PF-23D	+	RF-1	+
PF-25A	+	RF-1B	+
PF-26	+	RF-5	-
PF-26G	+	RF-5B	+
PF-27B	+		

*Positive growth was determined by increasing turbidity compared to controls.

Table 2. Growth of Fungi & Yeast Isolates at Varying Low pH Conditions.

Fungal Identification	**Medium pH**				
Strain Number	4	3	2.5	2	1.5
49-1c	+	+	-	-	-
52-1	+	+	+	-	-
52-1b	+	+	+	-	-
53-11A	+	+	-	-	-
53-11B	+	+	-	-	-
53-2B	+	+	-	-	-
53-2c	+	+	-	-	-
53-3DA	+	+	+	-	-
53-6A	+	+	+	-	-
53-6C	+	+	+	-	-
65-1	+	+	-	-	-
PF-41A	+	+	-	-	-
PF-46	+	+	-	-	-
PF-46A	+	+	+	-	-
PF-46B	+	+	-	-	-
PF-49	+	+	-	-	-
PF-53	+	+	+	-	-
PF-55	+	+	+	-	-
PF-14B	+	+	-	-	-
PF-21A	+	+	+	-	-
PF-23C	+	+	-	-	-
PF-23D	+	+	-	-	-
PF-25A	+	+	-	-	-
PF-26	+	+	+	-	-
PF-26G	+	+	+	-	-
PF-27B	+	+	+	-	-
PF-28	+	+	+	-	-
PF-30B	+	+	-	-	-
PF-36	+	+	+	-	-
PF-39	+	+	-	-	-
PF-5	+	+	-	-	-
PF-9C	+	+	-	-	-
RF-1	+	+	+	-	-
RF-5	+	+	+	-	-

Table 3. Identification of Fungal & Yeast Isolates using BIOLOG™

Fungal & Yeast	Biolog ID
49-1c(tan)	*Penicillium chrysogenum*
52-1	Fungi-No ID
52-1b	*Fusarium sp.*
53-11A	*Penicillium spp.*
53-11B	*Penicillium spp.*
53-2B	Penicilium brevicompactum
53-2c	*Paecilomyces variotii*
53-3DA	Fungi-No ID
53-6A	*Paecilomyces variotii*
53-6C	*Eupencillium brefeldianum*
65-1	*Candida parapsilosis*
PF-41A	Fungi-No ID
PF-46	*Fungi-No ID*
PF-46A	Fungi-No ID
PF-46B	Fungi-No ID
PF-49	Fungi-No ID
PF-53	Fungi-No ID
PF-55	Yeast-No ID
PF-14B	*Paecilomyces lilacinus*
PF-14B	*Paecilomyces lilacinus*
PF-21A	*Penicillium citrinum*
PF-23C	Fungi-No ID
PF-23D	*Penicilium brevicompactum*
PF-25A	*Penicillium simplicissium*
PF-26	*Penicillium simplicissium*
PF-26G	*Penicillium simplicissium*
PF-27B	Yeast-No ID
PF-28	*Aspergillus sydowii*
PF-30B	Fungi-No ID
PF-36	*Trichoderma harzianum*
PF-39	*Candida sp.*
PF-5	*Paecilomyces lilacinus*
PF-9C	*Paecilomyces lilacinus*
RF-1	*Candida Fluvialitis*
RF-5	*Candida Fluvialitis*

*No ID means were identified as fungi or yeast w/microscopy but did not meet Biolog™ criteria for speciation.

Table 4. Respirometric rates in Select Fungal & Yeast (Y) Isolates

Fungal Isolate	Protein	O_2 (ul/hr/g)	CO_2 (ul/hr/g)
PF-46A	.088g	-2101.89	102.131
PF-26	.048g	-1234.77	280.039
PF-30C	.050g	-6.321	2.025
PF-21A	.127g	-2.615	1.027
52-1	.079g	-245.95	247.26
PF-32	.125g	-0.1232	0.881
65-1 Y	.86g	0.565	-1.167
RF-5	.284g	-6.648	5.876
PF-39 Y	.32g	-1.074	0.318

Respirometric rates are expressed as microliter of gas (O_2 or CO_2) (ul) produced/consumed per hour (hr) per gram wet weight (g) of fungal isolate. CO_2-C evolved (mg C kg -1 dry soil) Protein is mass/gm wet weight in fungus inoculum.

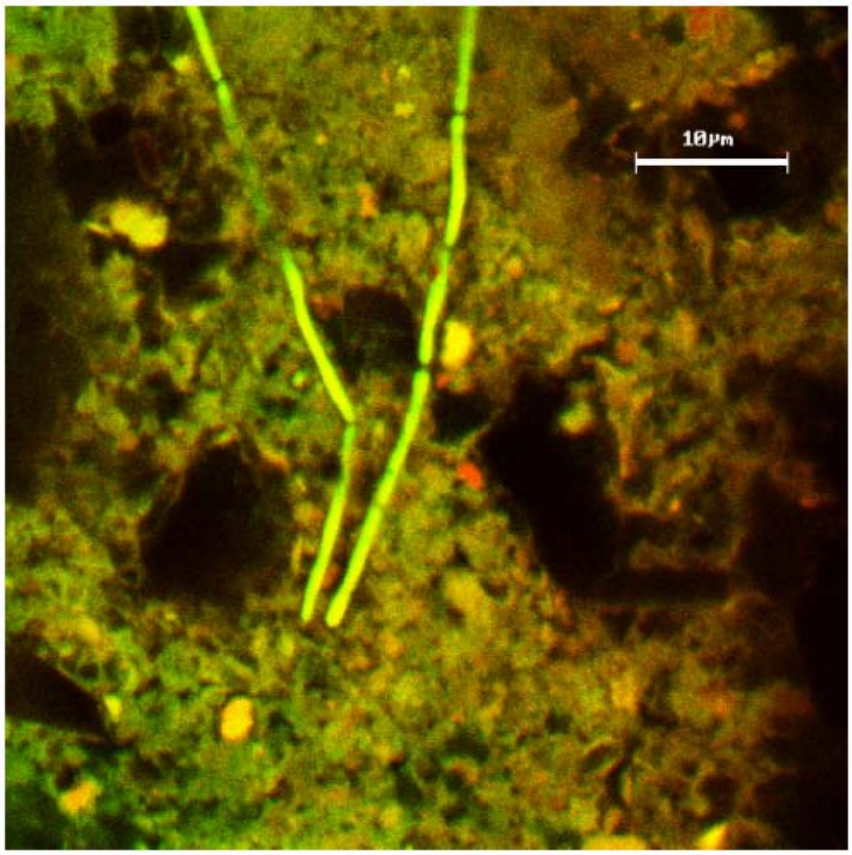

Figure 1. Laser Scanning Confocal Image of fungal hyphae in fresh polyaromatic hydrocarbon-contaminated CZOR lagoon sample using epifluorescent stain BAClight for fungi.

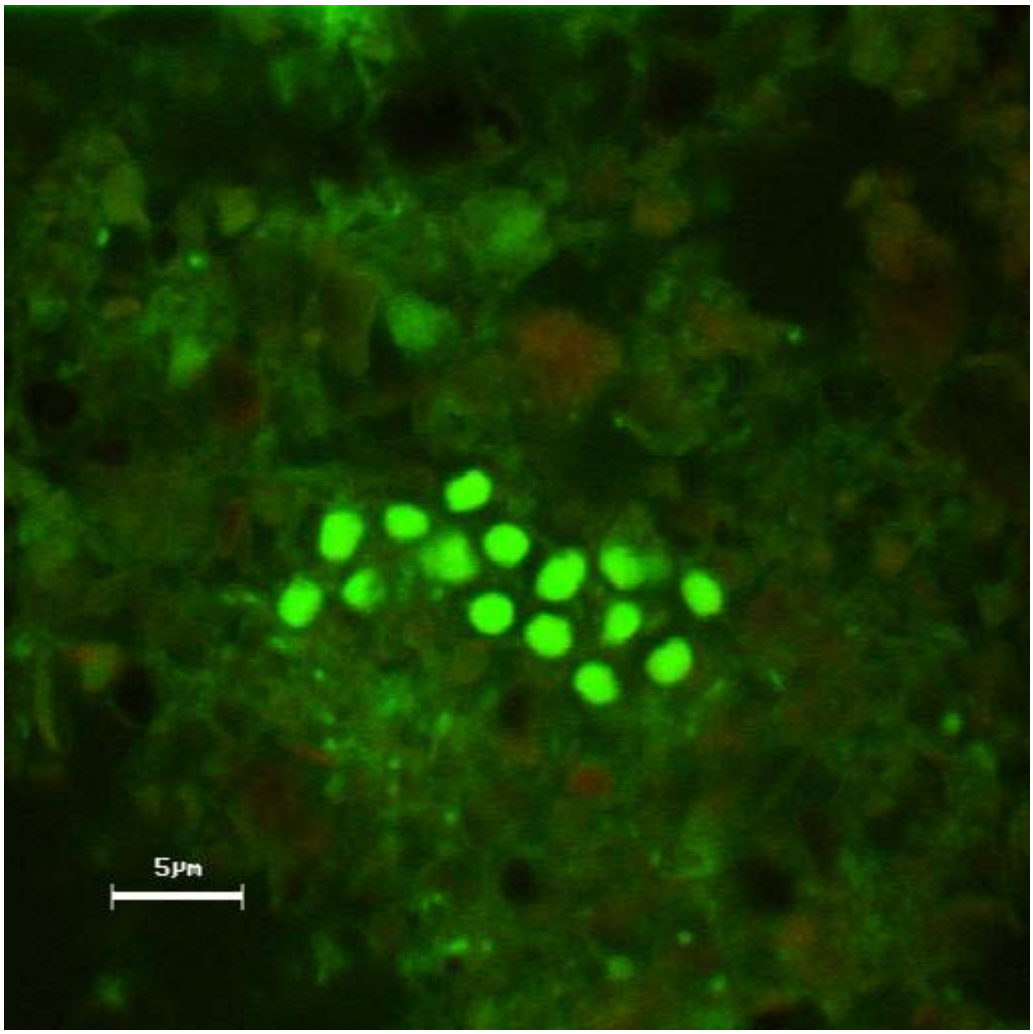

Figure 2. Yeast-like organisms stained with fluorescein identified in the sludge lagoon material.

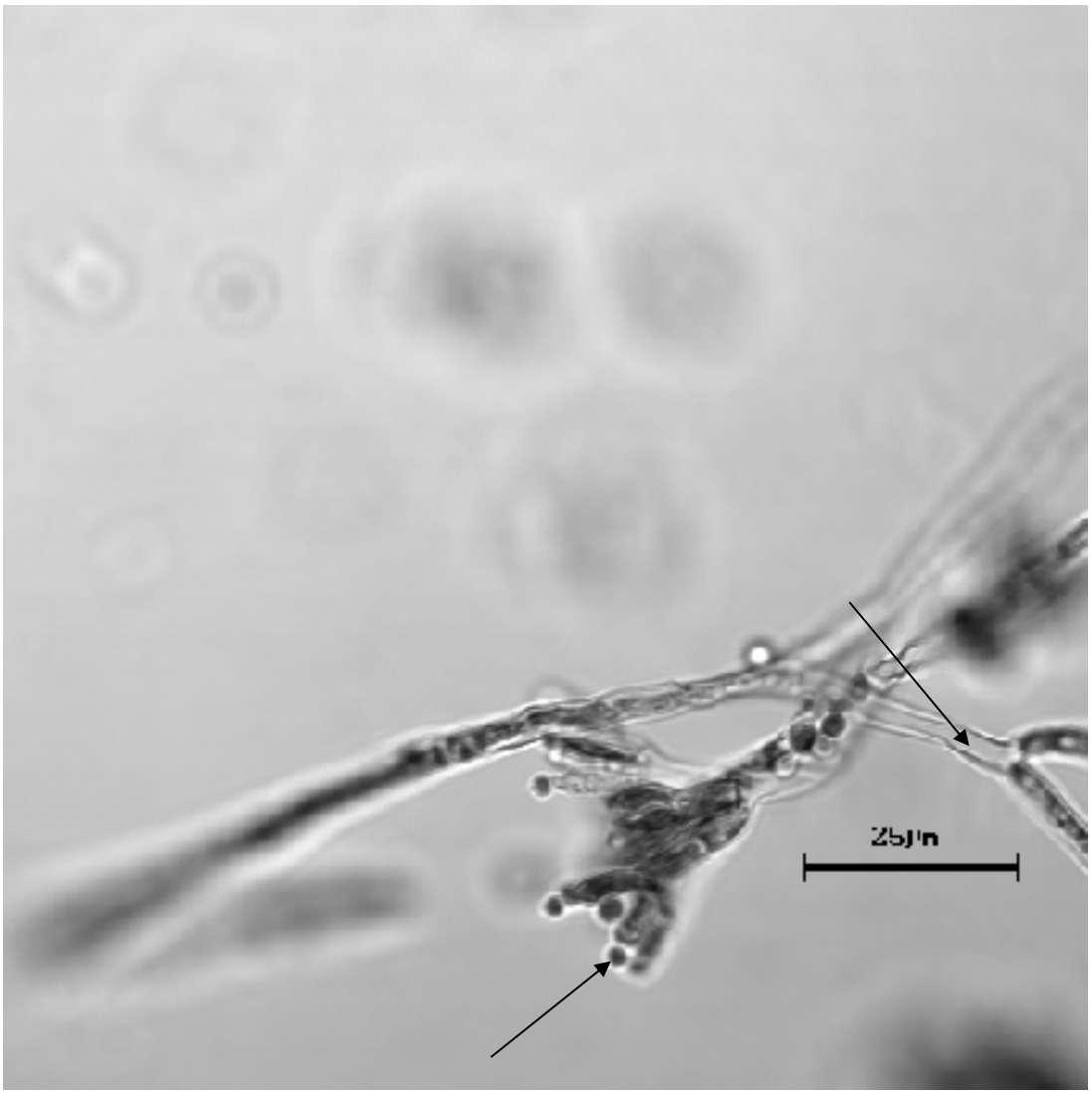

Figure 3. Confocal Image of Fungal Isolate PF-26 (Live Culture) identified by BIOLOG™ as *Penicillium simplicissium.* Arrows point to identifying structures. Scale bar 25 µm.