FIRST DIVERSITY ASSESSMENT AND SYNTHETIC DYES REMEDIATION POTENTIAL OF AQUATIC MICROMYCETES FROM AIN SKHOUNA WETLAND, WESTERN STEPPE OF ALGERIA

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Abstract. A first exploration of aquatic microfungal diversity was conducted in Ain Skhouna wetland, a part of Echott Ech Chergui (Algeria), the second largest chott in North Africa. Fungal strains isolation was carried out from water and sediment samples during 2019.Physico-chemical parameters (pH, temperature, and salinity)were measured*in situ* as well. Firstfungal strains identification wascarried out by cultural and structural features. A total of 14 monoclonal fungi strains belonging to 7 genera were distinguished where the majority of which belong to the phylum Ascomycota. Among these, the genus *Halobyssothecium* that was recorded for the first time in Algeria. Occurrence percentage frequency of different taxa showedhigher density of microfungal colonies in sediment compared to water. Through molecular barcoding successfully completed for 11 strains by Internal Transcribed Spacer (ITS) region sequencing, 5 strains were assigned to the species level and 6 to the Section or Complex within the correspondent genus. Authenticated strains based on ITS barcode were then screened for extracellular laccases production for mycoremediation assay, where 8 of them were laccases positive with strong activity exhibited by *Aspergillus sp.* AS02 and *Penicillium sp.*AS13 strains. All positive laccases strains were further investigated for their synthetic dyes decolorization ability using « Plate Volume Method » (PVM) to evaluate Congo Red, Brilliant Blue, and Malachite Green dyes decolorization activity at three concentrations, highlighting dyes biodegradation mechanisms used by each strain. Dyes decolorization seems more related to fungal laccases activity than biosorption or accumulation mechanisms. *Aspergillus sp.* AS02 and *Penicillium sp.* AS13 strains seem to be good dyes decolorizing candidates by biodegradation. Furthermore, *Fusarium acuminatum* AS07 showed significant biosorption activity, while *Penicillium spp*. generally expressed a good dyes accumulation potential. Therefore, identified and studied fungal strains could be used as promising tools for bioremediation of wastewater dyes and industrial effluents enclosing synthetic dyes.
Keywords: *microfungi, isolation, laccases, mycoremediation, Plate Volume Method*

Introduction

Micromycetes are ubiquitous and very widespread in nature (Li, 2016). Despite the relatively few research data they are known to be among the most diverse organisms in the world (Hawksworth, 2001; Blackwell, 2011; Hawksworth and Lücking, 2017). Schmit and Mueller (2007) gave a minimal estimate of 600 000 microfungi, 3000 occurring in aquatic habitats (Abdel-Aziz, 2008). Taxonomically, all fungal phyla arerepresented in aquatic environments (Tsui et al., 2016). This group constitutes a

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significant proportion of the biota and plays important roles in ecological processes. In aquatic ecosystems, they are mainly present in lotic systems, mangroves and wetlands, mainly decomposers of leaves, wood, detritus and other recalcitrant organic particles (Gulis et al., 2008; Seena et al., 2008). Major groups of Micromycetes occur in wetlands, but some are more represented than others, and associated to specific wetland conditions (Stephenson et al.,2013).

Fungi as well as other microorganisms are traditionally classified on the basis of their cultural, structural and biochemical features. However, molecular data sets like DNA/RNA based molecular markers have allowed the advancement of a more expected classification and improved appreciative fungal diversity (Shamim et al., 2017). The nuclear ribosomal transcribed internal spacers (ITS) region has a long history of use as a molecular marker for species-level identification in ecological and taxonomic studies of fungi (Hibbett et al., 2011) and phylogeny analysis. It offers several advantages over other species-level markers in terms of high information content, ease of amplification and multi-copy number per genome (Datta et al., 2011; Thangadurai et al., 2016), thus it has recently been designated the official barcode for fungi (Schoch et al.,2012; Kõljalg et al., 2013; Li, 2016). According to Karsch-Mizrachi et al. (2018), more than 1 000 000fungalITS sequences are available in the International Nucleotide Sequence Database Collaboration (INSDC: GenBank, ENA, and DDBJ) (http://www.insdc.org/). Other web-based databases are available includingUser-Friendly Nordic ITS Ectomycorrhiza: Unite (Nilsson et al., 2019) which holds only sequences from the ITS region of fungi and Mycobank (Robert et al., 2013).

Microfungi are used in food, paper, drug and dye processing industries, for enzymes, organic acids and antibiotics production (Tkacz and Lange, 2004; Bérdy, 2005) and serve as experimental organisms (Hyde et al., 2019). It has become a subject of great importance as new fungi and their associated biomolecules are identified (Prasad, 2017). The majority of these involved organisms are filamentous with minor proportion of yeasts (Bills and Gloer, 2016). Additionally, along bacteria, they are known as the principal environment xenobiotics decomposers, but fungi are stronger in dyes biodegradation/decolorizationthanks to their importantdyes degrading enzymes production, like laccases and peroxidasesand as absorbent (Sharma et al., 2016; Singh, 2017). Laccases are a copper-containing polyphenol oxidase acting on a wide range of substrates (Buddollaet al., 2008). They areefficient decomposers of synthetic dyes by oxidative pathway(Tauber et al., 2005). Actually, fungi are being increasingly used in bioremediation, called “mycoremediation” (Prasad,2017), to degrade or sequester environmental contaminants, particularly synthetic dyes which are nowadays among the major environmental pollutants (Vala and Dave, 2017), since traditional physico-chemical pollutants removal techniques are expensive and less effective (Deshmukh et al., 2016). Thus, study of novel fungi strains from unusual or unexplored habitats for biotechnological potential uses is equally very important for ecological and industrial perspectives.

Hitherto, a few scientific data are available in Algeria concerning aquatic fungi diversity and its biotechnological potentials, especially from wetlands. Therefore, the current work aims to highlight the microfungal diversity from water and sediment samples of Ain Skhouna wetland being the main part of Chott Ech Chergui wetlands complex classifiedby Ramsar convention since 2001 (Ramsar, 2019). Fungal isolates were initially characterized by classical methods involving cultural and structural features, which allowed to calculate the percentage frequency of occurrence of different

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taxa recovered, while molecular identification was performed using internal transcribed spacer (ITS) marquers. Furthermore, all genetically characterized strains deposited in the Genbank database were screened for production of laccases enzymes. Finally, strains showed positive laccases activity were tested, on solid mediaunder standard culture conditions of pH and temperature, for their bioremediation ability toward some synthetic dyes, namely Congo Red (CR), Brilliant Blue (BB) and Malachite Green (MG), at different initial concentrations, while highlighting other mechanisms used by each fungus to decolorize the medium. This work is the first report on Micromycetes diversity and its biotechnological potential from this specific area.

Materials and methods

Study area

This research was carried out in Ain Skhouna wetland, a part of Echott Ech Chergui wetlands complex (*Fig.1*). This wide area is an extensive closed depression containing permanent and seasonal saline, brackish and freshwater lakes, and pools, as well as hot springs. Situated in the western steppe part of Algeria, Ain Skhouna is about 350 Km far from the city of Oran above sea level for about 1000 meters.

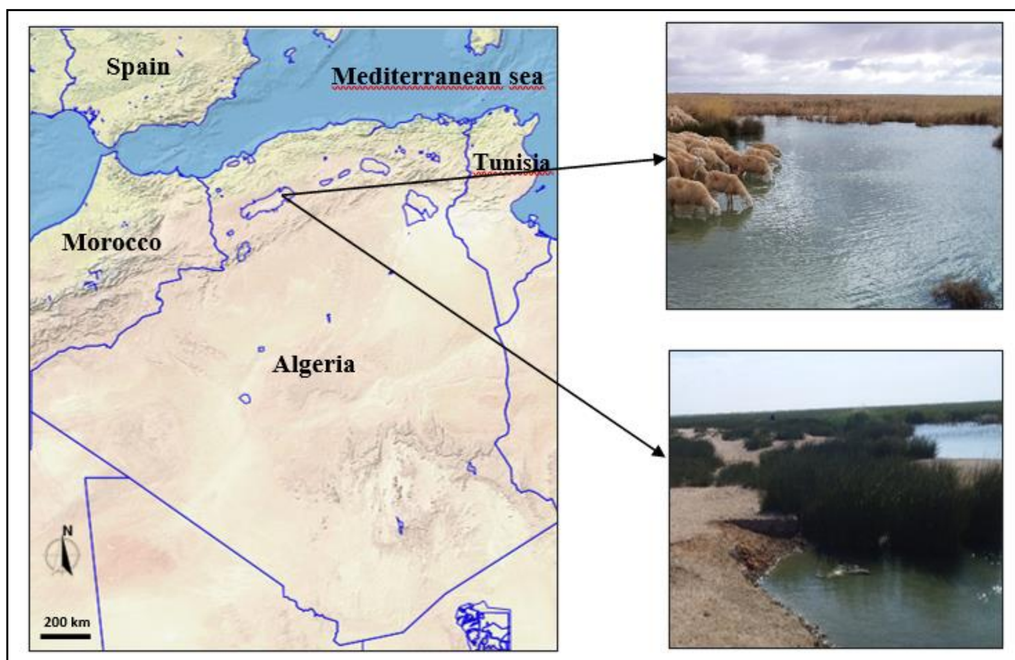

Figure 1. *Geographical location of the studied area*

Ain Skhouna is characterized by semi-desertic and stepic climate where temperatures average ranges from 5.1 to 26.3°C according to ONM (2015) (*Table 1*). This ecosystem is particular with salted calcimagnesic and chlorosulforic spring water composition(Remini, 2010),water-birds and vegetation (Benslimane et al., 2015), hence its classification by Ramsar convention in 2001 (Ramsar,2019). Its surrounding aquatic and terrestrial vegetation are reduced and, generally, consists mainly of areas of Alfa and Mugwort (Chih).

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Table 1. *Rainfall and temperature monthly average of Ain Skhouna 2000-2014 (ONM 2015)*

Parameter	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Total
Rainfall (mm)	18.26	20.91	23.97	19.04	21.49	4.74	2.92	6.3	8.74	18.26	23.6	18.39	186.62
Temperature (°C)	5.1	6.7	9.9	12.8	17	22.6	26.3	25	21.1	15.2	9.7	6.0	14.78

Period and samples collection

Monthly marginal surface water and submerged sediment samples were collected aseptically from 4 locations (*Table 2*) during 2019 from January to December in Ain Shkouna wetland. The geographical location of each sampling site was recorded using digital GPS (GARMIN). Within each site, two samples of both water and sediment were collected from the same point, totaling sixteen samples monthly. They were stored into sterile plastic containers using a sterilized spatula to collect sediment samples(Mohamed-Benkada,2006) and thenimmediately taken for analyzing to the Laboratory of Aquaculture and Bioremediation (*AquaBior*), located at Ahmed Ben Bella Oran1 University, Algeria.

Table 2. *Ain Skhouna wetland sampling site*

Site	Geographic coordinates	
	Lat	Long
S1	34,28,01 N	00,49,08 E
S2	34,30,08 N	00,50,44 E
S3	34,29,75 N	00,50,95 E
S4	34,28,11 N	00,43,38 E

Water samples physico-chemical parameters

Water samples were analysed for pH, salinity, temperatureand dissolved oxygen concentrationon site, using amultiparameter.

Water samples treatment

Decimal serial dilutions using 1 mL of water samples were aseptically carried out first in 9 mL of sterile distilled water (dilution 10^{-1}). The latter is used to obtain respectively 10^{-2} than 10^{-3} dilution solution factor. From each solution, a volume of 0,1 mL was plated in triplicate on Potato Dextrose Agar (PDA) media Petri dishes by spread plate technique. The Petri dishes were incubated at 25°C for 5 to 7 days.

Sediment samples treatment

One Gramm of each sample was aseptically diluted in 9 mL of sterile distilled water (dilution 10^{-1}), which is successively diluted using decimal factor to 10^{-2} and 10^{-3}. A volume (0.1 mL) from each dilution was plated aseptically in triplicate on PDA media Petri dishes by spread plate technique. Petri dishes were incubated at 25°C for 5 to 7 days.

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Fungal colonies total count

Estimation of the total number of Colony-Forming Unit (CFU) was done visually.This technique allowscells microorganisms enumeration in small volume samples, spread over the surface of an agar plate, resulting in the formation of discrete colonies -after appropriate incubation period- expected issued from a unique cell, distributed evenly across the agar surface when the appropriate concentration of cells is plated (Sanders, 2012).

Purification and identification of fungal strains

From each morphologically distinct fungal colony, aseptic subculturing of mycelium was carried out in Petri dishes on Sabouraud Dextrose Agar (SDA) media and then incubated at25°C, until agar surface invaded. Purified strains were identified using both macro and microscopic features referring to McClenny (2005), Leslie and Summerell (2008),Walsh et al.(2018) and Houbraken et al.(2020).

Percentage frequency of occurrence of fungal isolates

We calculated the percentage frequency of occurrence of each taxon isolated from the sampling sites, for water and sediment matrix, using the formula:

$$\% F = \frac{A}{B} \times 100 \quad \text{(Eq.1)}$$

where:
% *F*– Percentage frequency of occurrence for isolated fungal taxa,
A – total number of CFU of specific monoclonal fungal taxa,
B – CFU total number of whole fungal isolated colonies.

Molecular barcoding

DNA isolation, Internal Transcribed Spacer (ITS: ITS1-5.8S-ITS2) region amplification, and DNA sequencing were performed by Gene Life Science society (https://genelifesciences.com/). DNA was extracted from Saboreaud Dextrose Agar (SDA) cultures using NucleoSpin Plant II kit (Macherey-Nagel Germany). Specific DNA of each fungus was amplified by PCR using following primers: Forward ITS1-F (5’-CTTGGTCATTTAGAGGAAGTAA-3’) and Reverse ITS4-R(5’-TCCTCCGCTTATTGATATGC-3’) according to Gardes and Bruns(1993). PCR was carried out using the following protocol: initial denaturation at 95°C for 05 mn, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 45 s; a final elongation step was performed at 72°C for 07 mn. Following components were added for each 25 µL total reaction volume: Taq DNA polymerase Promega 1U, Taq polymerase buffer Promega 5 µL (1X), $MgCl_2$ 1.5 µL (1,5 mM), dNTP0,2 µL (0,2mM), Forward primer 1µL (0.5 µM), Reverse primer1µL(0.5 µM), Template DNA2 µL, and ultra-pure water 10 µL. The obtained PCR product was purified using NucleoSpin® Gel and PCR Clean-up Macherey-Nagel (Germany) Kit and then stored at -20°C. The resulting amplicon was visualized by horizontal electrophoresis on 1.5% (w/v) agarose gel using Bio-Rad Gel doc system (USA).The isolated and purified PCR products were sequenced using the Sanger technique (Sanger et al., 1977) using the

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Big Dye v3.1 kit from Applied Biosystems and the PCR primers used for the amplification of the fragments of interest (ITS).

Taxonomy analyses

The obtained ITS sequences were compared with the available sequences in GenBank database (Zhang et al., 2000) using the NCBI n-BLAST search program of the National Center for Biotechnology Information (NCBI).

Data availability

All ITS region rRNA gene sequences generated from this study were deposited at NCBI GenBank database.

Screening of fungal strains for laccases production

A screening ofisolated fungi laccases potential productionwas performed on ITS barcode authenticated strains (11 strains). Tekere et al. (2001) method was carried out using PDA medium supplemented with 0.2 g/Lof Bromophenol Blue and 0.01% Chloramphenicol (to avoid bacterial growth). The pH was adjusted to 5.0. The plates were inoculated with 5 mm mycelial fungal studied strains plugs of 7 days old in triplicates for each one. Positive controls made of un-supplemented PDA plates inoculated as previous were used. Negative controls were also made with PDA plates amended only with Bromophenol Blue (0.2 g/L). Petri dishes were incubated at 25°C for 7 days. The presence of clear halos around Laccases producing colonies indicates dyes oxidation. Laccases production was expressed as Activity Ratio (AR) which corresponds to the activity diameter (clear halo in cm) divided by the colony diameter (cm) and appreciated as follows: AR > 2 (+++, high laccases production), 2 ≥ AR>1.5 (++, moderate laccases production), 1.5 ≥ AR> 0 (+, low laccases production), AR = 0 (-, no activity) (Jaouani et al., 2014).

Dyes decolorization assay

The isolates showing laccases production were screened further for dyes removal ability.The decolorization of one azo dye Congo red (CR) and two triphenylmethane dyes Brilliant Blue G-250 (BB) and Malachite Green (MG), largely used in textile industries, was tested at 3 different concentrations (100, 200 and 500 ppm) as described by Pointing (2001). The experimental conditions used in laccases production test above were repeated herein, except the incubation period which was extended to 16 days (Kumar et al., 2020). The mycelial growth as well as decolorized zone diameters produced by different strains was measured at incubation end. Dyes percentage decolorization was appreciated using « Plate Volume Method » (PVM) (Shah et al., 2021)**,**andestimated by the following equation:

$$\% \, Decolorization = \frac{(Diameter of decolorized \ region)^2}{(Diameter \ of \ solid \ medium \ plate)^2} \times 100 \qquad \text{(Eq.2)}$$

Mycelial growth diameter,decolorized zone diameter and decolorization percentage were calculated. The decolorization activity percentagewas appreciated as follows: 70 to 90% = strong decolorization (+++); 50 to 69% = moderate decolorization (++); 20 to 49% = weak decolorization (+) and less than 20% = no activity (−). Moreover, based on

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the report of Singh (2017)**,** other eventual dyes removal mechanismsassociated with the enzymatic biodegradation mechanism were noted for each studied fungal through direct observation of mycelium and fungal colony coloration along the experiment, without calculating the amount or percentage of dye removed through each mechanism. Solís et al. (2012) and Singh (2017) categorized dye removal process mediated by fungi into biosorption, bioaccumulation and biodegradation, which can occur simultaneously in one dye removal process (Park et al., 2007; Asses et al., 2018)**,** but as a primary mechanism of decolorization, dye should be adsorbed (adsorption) on the new formed hyphae cells (hyphal elongation) surface (Knapp et al., 1995; Kaushik and Malik, 2015). In biodegradation, molecules of dyes are broken-down through enzyme's action and fungal colony does not stain. As for biosorption, it involves the binding of solutes to the fungal surface biomass which can be noticed on the face of the colony while the bottom keeps its original color and not that of the dye. In contrast, accumulation occurs in the cell cytoplasm (Timková et al., 2018) wherethe fungal colony's reverse side looks stained by the tested dye, unlike the front side which retains its natural color.

Statistical analysis

Each experiment was conducted in triplicates. A Two-Way Analysis of Variance (ANOVA) was performed to identify the effect of the dyes concentrations on decolorized zone formation and, a Three-Way ANOVA to demonstrate highly effective strains, using statistical package R version 4.1.1 (R Core Team, 2021). Means were compared using Tukey's HSD ($\alpha = 0.05$) post-hoc test.

Results

Water physico-chemical parameters

In *Table 3* are summarized*in situ* annual average water physico-chemical parameters results. The pH values slightly differ between sites, ranging from 6.9 to 7.8 with irregular monthly variations during the study period. The same pattern was shown for the salinity parameter ranging from 0.95 g/L to 2.3 g/L. Thus, we can classify the water quality as fresh to slightly brackish and neutral to slightly basic pH. Whereas overall monthly average of temperature values had significant monthly variation ranging between 36°C (site1) to 12.7°C (site 3). This average was highest at site 1 (31°C) and site 2 (25.6°C) with a close monthly variation due to their proximity to hot springs nearby (origin of the water of Ain Skhouna wetland), while at the remaining sites (3 and 4), further away from these showing a significant monthly variation, reached 22.5°C and 24.3°C, respectively. The dissolved oxygen concentrations (DO) showed a contradictory pattern compared to the temperature level and was ranged from 3.7 ppm to 14.3 ppm with a mean value of 7.6, 8.4, 9.7 and 9.4 from sites 1, 2, 3 and 4, respectively. Taking into account that cold water holds more DO than warm water, sites 3 and 4 were found to exhibit higher DO than sites 1 and 2.

Fungal colonies total count and community structure

Based on the isolation samplings, Micromycetes propagules density varied between the two compartments. The overall monthly average density was estimated to 629 CFU $1mL^{-1}$ in the water and 752 CFU $1g^{-1}$ in the sediment. The samples carried out in spring and fall months contained higher values both in water and sediment. During the winter

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and summer months, the greatest number of propagules was found in sediment samples. A common white-beige colony constituted a major number of propagules throughout the year for all sites in both water and sediment materials, whereas other colonies were more or less present.

Table 3. *Physico-chemical parameters of Ain Skhouna wetland water during 2019*

Site	pH			Salinity (g/L)			Temperature (°C)			DO (ppm)		
	min	max	M ± S	min	max	M ± S	min	max	M ± S	min	max	M ± S
S1	7.2	7.8	7.5 ± 0.2	1	1.3	1.2 ± 0.1	27.5	36	31 ± 3.1	3.7	11.2	7.6 ± 2.9
S2	7.2	7.7	7.4 ± 0.1	0.95	1.25	1.1 ± 0.1	19.8	33.2	25.6 ± 4.2	5.15	12.5	8.4 ± 2.3
S3	6.9	7.3	7.1 ± 0.1	1.5	2.3	1.9 ± 0.3	12.7	30.32	22.5 ± 5.3	6.2	14.3	9.7 ± 2.7
S4	7	7.55	7.2 ± 0.2	1.2	1.7	1.4 ± 0.2	14.4	32.2	24.3 ± 6.0	7.32	12.86	9.4 ± 2.1

The morphological and microscopic features showed that all isolated strains from Ain Skhouna wetland were able to produce spores and, thus, enabled their classification at the genus level. The microfungal community was constituted by 14 strains belonging to 7 genera (*Table 4*) and each of them was assigned a code number from AS01 to AS14, AS being the abbreviation of Ain Skhouna (study area). They were found mitosporic Ascomycetes so-called aquatic–terrestrial fungi: *Candida, Penicillium, Aspergillus, Cladosporium, Fusarium* and *Halobyssothecium*, except *Mucor* (Mucoromycetes).

Table 4. *Microfungal community structure of Ain Skhouna wetland*

Strain code	Genus	Strain code	Genus
AS01	*Candida*	AS08	*Mucor*
AS02	*Aspergillus*	AS09	*Halobyssothecium*
AS03	*Aspergillus*	AS10	*Penicillium*
AS04	*Aspergillus*	AS11	*Penicillium*
AS05	*Cladosporium*	AS12	*Penicillium*
AS06	*Penicillium*	AS13	*Penicillium*
AS07	*Fusarium*	AS14	*Cladosporium*

Percentage frequency of occurrence of fungal isolates

The total frequency percentage of occurrence of isolated Micromycetes taxa is presented in *Table 5*, and according to the sampling sites is depicted in *Figure 2*. *Candida* was the only and common genus of yeasts isolated from all samples, with the highest frequency of occurrence both in water and sediment. The remaining genera were molds fungi, so *Penicillium* genus, which was the most diverse among all taxa (5 strains), was frequent in all sites representing the second most important group in terms of abundance, followed by *Aspergillus* and *Cladosporium*, respectively. The remaining genera namely *Fusarium, Mucor* and *Halobyssothecium* were of low occurrence. However, for sampling sites, sites 1 and 2 were the most diverse and site 3 was the least. *Candida*, *Penicillium*, *Aspergillus*, *Cladosporium*, and *Fusarium spp.* were common in all sampling sites, except

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for what concerns *Mucor* taxa, absent in site 3, while *Halobyssothecium sp.* was recovered only from site 1 and site 2. However, although the community of Micromycetes differed between sites, it was homogeneous between the water column and the sediment (see *Table A1* and *Table A2*).

Table 5. *Percentage frequency of occurrence of microfungal taxa isolated from Ain Skhouna wetland*

Sample	Site	Total CFU count average	Individual fungal taxa CFU count average						
			Can	*Pen*	*Asp*	*Cla*	*Fus*	*Muc*	*Hal*
Water	S1	224	162	27	16	7	3	4	5
	S2	179	123	22	19	9	1	3	2
	S3	102	70	12	11	5	4	0	0
	S4	124	82	15	10	6	4	7	0
Total CFU		629	437	76	56	27	12	14	7
% *F*		100	69,48	12,08	8,90	4,29	1,11	2,23	1,91
Sediment	S1	275	199	32	18	14	4	3	5
	S2	224	153	30	20	9	5	2	5
	S3	92	61	13	9	6	3	0	0
	S4	162	103	24	14	11	3	7	0
Total CFU		752	516	99	61	40	15	12	9
% *F*		100	68,62	13,16	8,11	5,32	1,20	1,60	1,99

CFU: Colony Forming Unit, % F: Percentage frequency of occurrence, Can: Candida, Pen: Penicillium, Asp: Aspergillus, Cla: Cladosporium, Fus: Fusarium, Muc: Mucor, Hal: Halobyssothecium

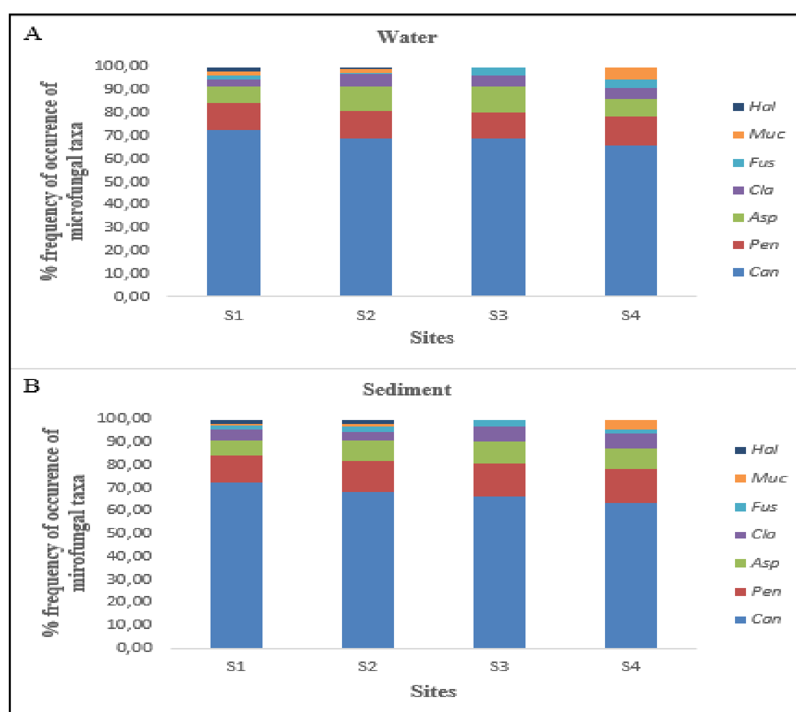

Figure 2. *Percentage frequency of occurrence of different microfungal taxa according to samplings sites. A: Water, B: Sediment, Can: Candida, Pen: Penicillium, Asp: Aspergillus, Cla: Cladosporium, Fus: Fusarium, Muc: Mucor, Hal: Halobyssothecium*

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Molecular barcoding

Out of 14 strains, we successfully amplified and sequenced the ITS marker for 11strains. Among them only 5 were identified at the species level, while the rest of strains (6) seemed to require further analysis since molecular marker was fully conserved with more than one species, according to megablast queries, and were assigned up to the phylogenetic section or complex within the genus. Strains taxonomic affiliations and their GenBank accession numbers are compiled in *Table 6*.

Table 6. *Identification of aquatic microfungal strains isolated from Ain Skhouna wetland based on ITS sequencing*

Strain code	Best megablast match (GenBank)	Section or Complex within the genus (Reference)	Identified species	GenBank accession number
AS02	100% conserved with: *Aspergillus niger*, *Aspergillus tubingensis*, *Aspergillus piperis*, *Aspergillus costaricensis* and *Aspergillus neoniger*	*Aspergillus* section *Nigri* (Meijer et al., 2011; Varga et al., 2011)	*Aspergillus sp.* strain AS02	OP009423
AS03	99.64% conserved with:*Aspergillusiranicus*isolate MUS_RUS1	*Aspergillus* section *Terrei* (Y.S. Zhang et al., 2018)	*Aspergillus iranicus*strainAS03	OP009424
AS05	100% conserved with: *Cladosporium cladosporioides*, *Cladosporium pseudocladiosporioides*, *Cladosporium xylophylum*, *Cladosporium acalyphae*, *westerdijkiae*, *Cladosporium vicinum*, *Cladosporium subuliforme*, *Cladosporium needhamense*, *Cladosporium inversicolor*, *Cladosporium funiculosum*, *Cladosporium europaeum*, *Cladosporium delicatulum* and *Cladosporium angustisporum*	*Cladosporium Cladosporioides* complex(Bensch et al., 2012)	*Cladosporium sp.*strain AS05	OP009425
AS06	100% conserved with: *Penicillium polonicum*, *Penicillium expansum*, *Penicillium aurantiocandidum* and *Penicillium glandicola*	*Penicillium s*ection *Penicillium* (Visagie et al. 2014; Houbraken et al., 2020)	*Penicillium sp.* strain AS06	OP009426
AS07	100% conserved with : *Fusarium acuminatum*	*Fusarium tricinctum* complex (Moretti, 2009)	*Fusarium acuminatum* strain AS07	OP009427
AS09	100% conserved with : *Lentithecium aff. carbonneanum*	*Halobyssothecium* clade (Calabon et al., 2021)	*Halobyssothecium carbonneanum* strain AS09	OP009428
AS10	99.65% conserved with: *Penicillium chrysogenum* and *Penicillium rubens*.	*Penicillium* section*Chrysogena*(Visagie et al., 2014; Houbraken et al., 2020)	*Penicillium sp.*strain AS10	OP009429
AS11	100% conserved with : *Penicillium commune* isolate AY109	*Penicillium s*ection *Fasciculata* (Visagie et al., 2014; J. Houbraken et al., 2020)	*Penicillium commune* strain AS11	OP009430
AS12	100% conserved with : *Penicillium commune* isolate UFMGCB	*Penicillium s*ection *Fasciculata* (Visagie et al., 2014; Houbraken et al., 2020)	*Penicillium commune* strain AS12	OP009431
AS13	100% conserved with: *Penicillium fellutanum*, *Penicillium janczewskii*, *Penicillium arizonense*, *Penicillium canescens*, *Penicillium jensenii* and *Penicillium radiatolobatum*	*Penicillium s*ection *Canescentia* (Visagie et al., 2014; Houbraken et al., 2020)	*Penicillium sp.*strain AS13	OP009432
AS14	100% conserved with: *Cladosporium cladosporioides*, *Cladosporium pseudocladosporioides*, *Cladosporium oxysporum*, *Cladosporium austroafricanum*, *Cladosporium xylophilum*, *Cladosporium subuliforme*, *Cladosporium verrucocladosporioides* and *Cladosporium tenuissimum*	*Cladosporium cladosporioides* complex (Bensch et al., 2012)	*Cladosporium sp.*strain AS14	OP009433

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Penicillium genus was the most diverse (5 strains) where AS06, AS10 and AS13 strains were described as *Penicillium spp.,* while AS11 and AS12 were two distinct strains of *Penicillium commune*. *Aspergillus* was the second diverse group (3 strains) with only two successfully sequenced strains. The first one encoded as AS02 was closely similar to many species of the *Aspergillus Nigri* section (the black aspergilli), and the second strain (AS03) was confirmed as *Aspergillus iranicus.* Both strains of the *Cladosporium* genus, AS05 and AS14 were not assigned to the species level as long as they showed 100% identity with many species belonging to the *Cladosporium cladosporioides* complex which includes the well-known *Cladosporium cladosporioides* species. As for the unique strain of the *Fusarium* genus (AS07), it was described as *Fusarium acuminatum* (Teleomorph: *Gibberella acuminata*) located in the *tricinctum* species complex (FTSC). Finally, BLASTn search for the strain AS09 indicated 100% similarity to *Lentithecium aff. carbonneanum*, while it should be named as *Halobyssothecium carbonneanum*, according to the currently nomenclature carried out by Calabon et al. (2021). It should be noted that we failed to match the ITS results by those of morphological observations due to the well documented confusion existing between species of *Penicillium*, *Aspergillus* and *Cladosporium* genera.

Based on isolation work and classical and genetic identification, *Figure 3* below shows the morphological and microscopic aspect of some strains isolated during the current study.

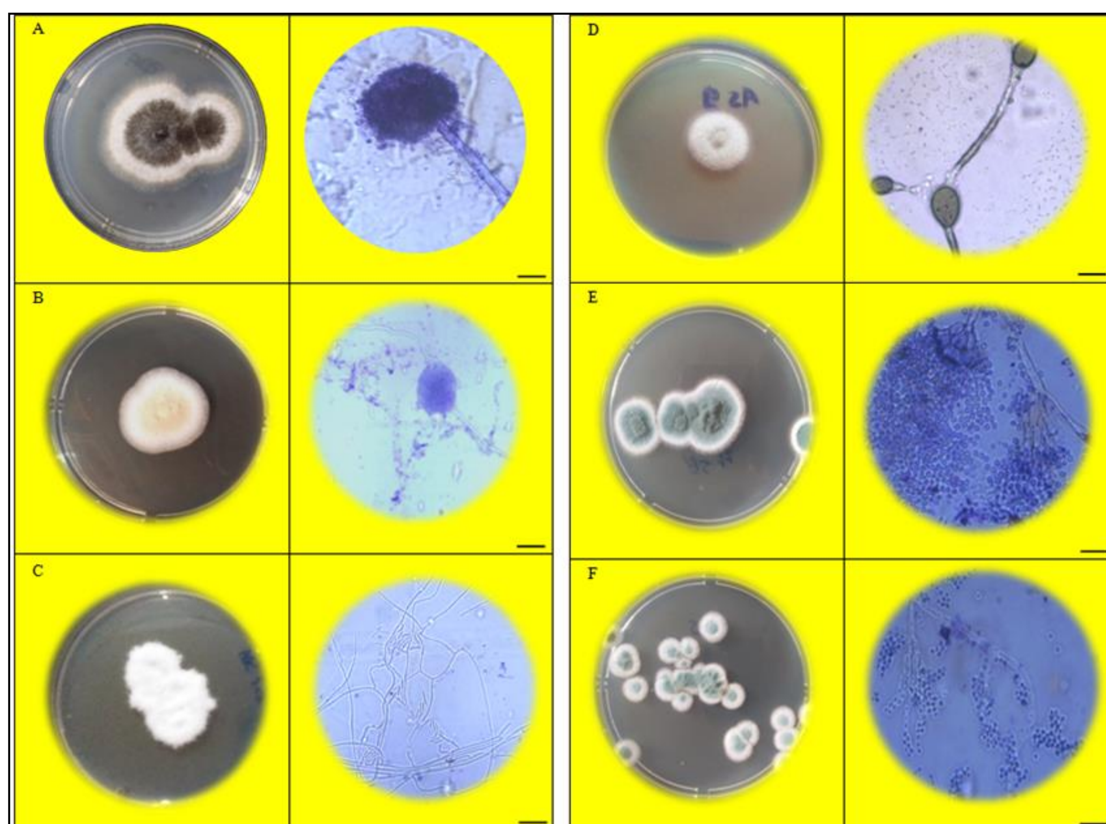

Figure 3. *Macroscopic and microscopic aspect of some microfungal strains isolated from Ain Skhouna wetland. A : Aspergillus sp. AS02, B : Aspergillus iranicus AS03, C : Fusarium acuminatum AS07, D : Halobyssothecium carbonneanum AS09, E : Penicillium commune AS11, F : Penicillium sp. AS13. Scales bars : 25 µm*

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Screening of fungal strains for laccases production

The results of laccases production test are shown in *Table 7*. Two strains, namely AS02 and AS13 showed high laccases production, while AS03, AS07 and AS11strains exhibitedmoderate production. The remaining strainsdisplayed a weak or negative laccases test. Strain AS06 produces a red diffusible pigment in the culture medium, thus it was not possible to verify the test.

Table 7. *Laccases activity ratio of microfungal strains from Ain Skhouna*

Strain code	Fungus	Laccases activity ratio
AS02	*Aspergillus sp.*	+++
AS03	*Aspergillus iranicus*	++
AS05	*Caladosporium sp.*	+
AS06	*Penicillium sp.*	*
AS07	*Fusarium acuminatum*	++
AS09	*Halobyssothecium carbonneanum*	+
AS10	*Penicillium sp.*	+
AS11	*Penicillium commune*	++
AS12	*Penicillium commune*	-
AS13	*Penicillium sp.*	+++
AS14	*Caladosporium sp.*	-

AR: activity ratio. −: no activity; +: AR < 1; ++: 1 < AR < 2; +++: 2 < AR < 3; *: not verified

Dyes decolorization assay

Mycelial growth

The diameter of mycelial growth expressed as mean values of three replicates at the 16th day of incubation of studied fungal strains is displayed in *Table 8*. All screened strains showed well growth on PDA containing CR and BB at 100, 200 and 500 ppm dye concentrations. Moreover, in comparison with the positive control, it was found that the CR and BB dyes had a good effect on the mycelial growth of the AS07 strain for the three concentrations tested. However, for Malachite green (MG) dye, only AS02 grows well in all concentrations, while *Penicillium spp. viz.* AS10, AS11 and AS13 exhibited a fair growth, when the remaining strains showed weak to total growth inhibition.

Table 8. *Mycelial growth (mm) of microfungal strains of Ain Skhouna at the 16th day of incubation*

Dyes / Strains	CR			BB			MG		
	100 ppm	200 ppm	500 ppm	100 ppm	200 ppm	500 ppm	100 ppm	200 ppm	500 ppm
AS02	77,67	75,33	73,67	80,33	74,33	71,00	73,67	72	65,33
AS03	54,33	50,33	38,67	60,33	52,00	49,33	34,00	22,33	19,00
AS05	57,00	43,67	24,67	47,33	46,00	34,67	16,67	16,00	12,33
AS07	54,33	52,00	50,00	48,00	45,33	44,67	45,67	33,33	22,67
AS09	50,33	47,67	45,67	47,67	47,33	45,67	20,67	5,33	0,00
AS10	48,33	45,33	43,67	50,67	49,33	47,67	43,00	49,00	35,67
AS11	56,33	54,67	53,67	52,33	50,33	45,00	50,00	55,00	39,00
AS13	64,67	60,67	58,33	64,67	56,33	54,33	55,00	52,33	40,33

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Decolorized zone formation

The color of the three tested dyes stayed stable in negative controls throughout the experiment. The mean values of decolorized zones diameter of the three replicates at the end of incubation period are summarized in *Table 9*. The eight strains showed differences in the formation of a clear zone from each other for the same concentration of the respective synthetic dyes, as well as between different concentrations for the same dye. A general observation is the decrease in decolorized zone with as the dyes concentration increase. Maximum clear zone formation was reported by the AS02 followed by AS13 for CR at 100 ppm concentration, while most restricted clear zone (no activity) was observed for AS09 for MG at 200 and 500 ppm.

Table 9. *Decolorized zone (mm) formed by microfungal strains of Ain Skhouna at the 16th day of incubation*

Dyes Strains	CR			BB			MG		
	100 ppm	**200 ppm**	**500 ppm**	**100 ppm**	**200 ppm**	**500 ppm**	**100 ppm**	**200 ppm**	**500 ppm**
AS02	86,67	82,33	79,33	85,33	79,33	66,67	81,00	76,33	68,00
AS03	83,67	71,33	64,00	76,67	64,33	53,00	64,00	41,33	24,67
AS05	77,00	64,67	55,33	71,67	58,67	41,67	52,33	31,00	14,67
AS07	78,33	71,33	64,67	75,33	70,33	65,33	64,33	46,33	24,67
AS09	78,00	70,33	51,00	81,33	72,33	63,67	43,00	0,00	0,00
AS10	80,33	70,33	67,00	79,67	70,67	55,00	75,00	62,33	47,00
AS11	81,67	74,67	68,33	79,33	68,00	52,33	78,33	68,67	57,67
AS13	84,67	79,33	73,67	80,00	75,33	67,67	79,67	74,00	63,67

Percentage decolorization of dyes

Calculated percentage decolorization of the three dyes exhibited by positive laccases fungal strains after 16 days of incubation is depicted in *Figures 4, 5 and 6*. It was observed that all isolates were able to decolorize CR and BB at 100 ppm and 200 ppm concentrations from moderate to strong. In case of CR at 500 ppm only AS02 was more efficient (77.71%), whereas AS13 (67.00%), AS11 (57.66%), AS10 (55.43%) and AS03 (50.59%) were moderately efficient. No strain showed strong decolorization for BB 500 ppm and only three strains; AS13 (56.65%), AS02 (54.88%) and AS07 (52.71%) revealed moderate decolorization. Maximum decolorization was observed with AS02 for MG at 100 ppm (81.02%), followed by AS13 (78.37%), AS11 (75.77%), AS10 (67.61%), AS07 (51.14%) and AS03 (50.63%), while highest percentage removal of MG at 200 ppm was recorded by strains AS02 (71.95%) and AS13 (67.61%) showing a strong and moderate decolorization level, respectively. In case of MG at 500 ppm, only AS02 and AS13 were able to remove it at more than 50%.

Table 10 and*Table 11* represent the results of the Two-Way ANOVA test for decolorized zone formation and those of the pairwise comparisons between dyes concentrations by post-hoc TukeyHSD test, respectively.

Figure 7 represents the overall average decolorization percentage exhibited by tested microfungal strains, while *Table 12* and *Table 13* regroup the results of Three-Way ANOVA for decolorization ability and those of the pairwise comparisons between fungal strains by post-hoc TukeyHSD test, respectively. The Tukey’s test results are also shown in *Figure 8*.

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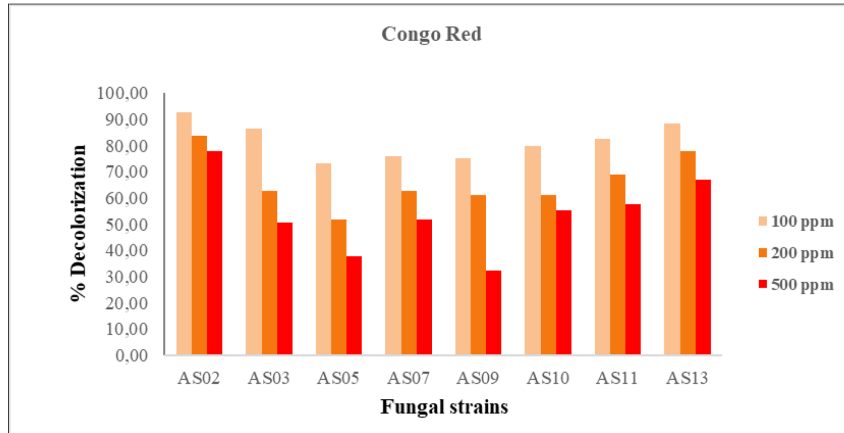

Figure 4. *PVM percentage decolorization of CR dye by microfungal strains from Ain Skhouna wetland*

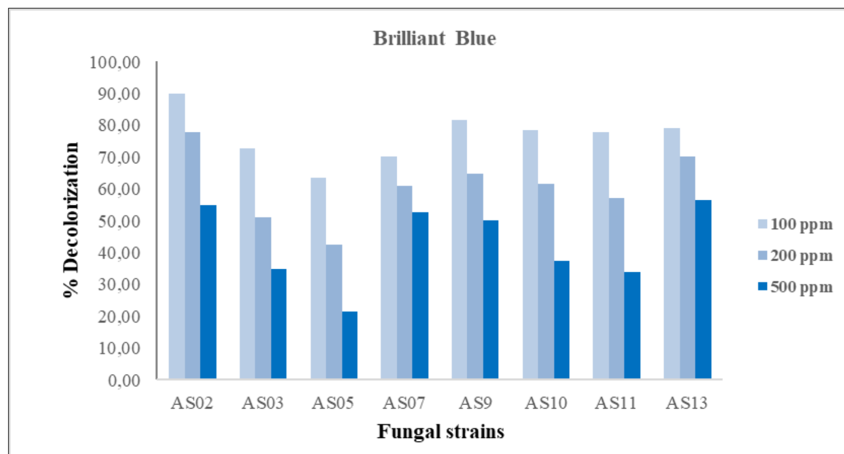

Figure 5. *PVM percentage decolorization of BB dye by microfungal strains from Ain Skhouna wetland*

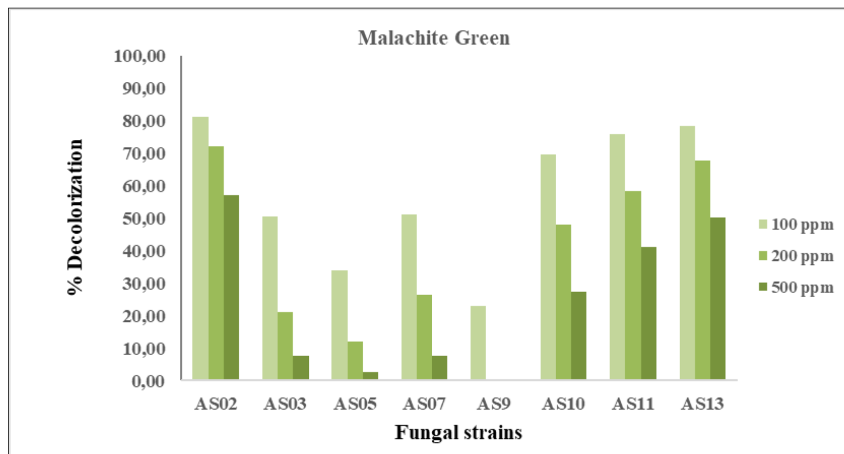

Figure 6. *PVM percentage decolorization of MG dye by microfungal strains from Ain Skhouna wetland*

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Table 10. *Two-Way ANOVA base for decolorized zone formation*

Factor	Df	Sum of squares	Mean of squares	F value	p value
Dye	2	6306	3153.1	16.117	2.22e-06 ***
Concentration	2	5818	2908.8	14.868	5.14e-06 ***
Dye :Concentration	4	433	108.3	0.554	0.697

Df: Degrees of freedom. Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Table 11. *Results of pairwise comparisons between dyes concentrations by post-hoc TukeyHSD test after Two-Way ANOVA decolorized zone formation*

Pair of dyes concentrations	diff	lwr	upr	p adj
200-100	-11.41792	-21.10971	-1.7261252	0.0170319
500-100	-22.01292	-31.70471	-12.3211252	0.0000026
500-200	-10.59500	-20.28679	-0.9032085	0.0289379

Df: Degrees of freedom. Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

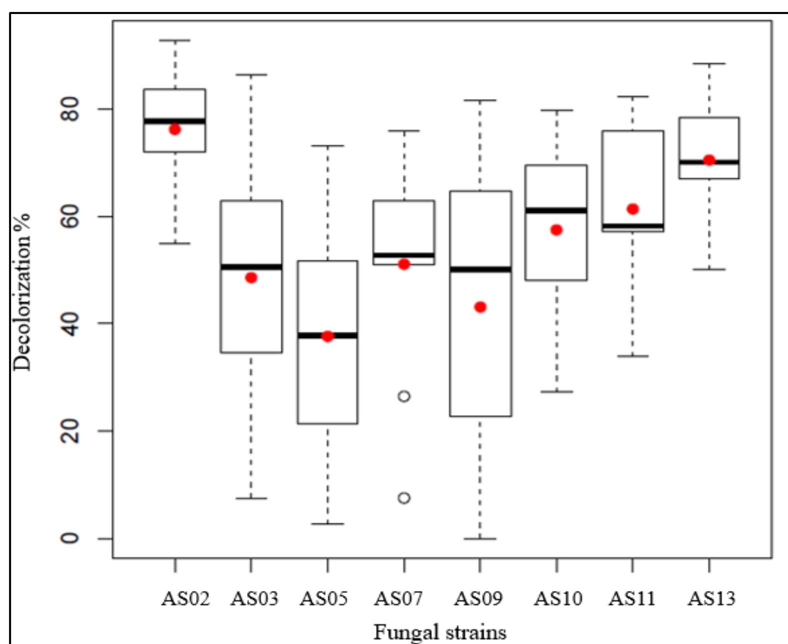

Figure 7. *Overall average decolorization percentage exhibited by microfungal strains from Ain Skhouna wetland*

Table 12. *Three-Way ANOVA base for decolorization ability*

Factor	Df	Sum of squares	Mean of squares	F value	p value
Strain	7	11163	1595	25.155	1.39e-09 ***
Dye	2	9505	4752	74.968	4.76e-11 ***
Strain:Dye	14	4736	338	5.337	0.000174 ***
Dye:Concentration	3	11086	3695	58.292	3.62e-11 ***
Strain:Dye: Concentration	21	852	41	0.640	0.847269

Df: Degrees of freedom. Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

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Table 13. *Results of pairwise comparisons between fungal strains by post-hoc TukeyHSD test after Three-Way ANOVA for decolorization ability*

Pair of fungal strains	diff	lwr	upr	p adj
AS03-AS02	-27,696667	-40,1272894	-15,266044	0,0000032
AS05-AS02	-38,697778	-51,1284005	-26,267155	0,0000000
AS07-AS02	-25,275556	-37,7061782	-12,844933	0,0000142
AS09-AS02	-33,241111	-45,6717338	-20,810488	0,0000001
AS10-AS02	-18,71	-31,1406227	-6,279377	0,0009676
AS11-AS02	-14,905556	-27,3361782	-2,474933	0,0112146
AS13-AS02	**-5,753333**	**-18,183956**	**6,677289**	**0,7827894**
AS05-AS03	-11,001111	-23,4317338	1,429512	0,1099989
AS07-AS03	2,421111	-10,0095116	14,851734	0,9977079
AS09-AS03	-5,544444	-17,9750671	6,886178	0,8116402
AS10-AS03	8,986667	-3,443956	21,417289	0,2881617
AS11-AS03	12,791111	0,3604884	25,221734	0,040567
AS13-AS03	21,943333	9,5127106	34,373956	0,000118
AS07-AS05	13,422222	0,9915995	25,852845	0,0279065
AS09-AS05	5,456667	-6,973956	17,887289	0,8232124
AS10-AS05	19,987778	7,5571551	32,4184	0,0004202
AS11-AS05	23,792222	11,3615995	36,222845	0,0000361
AS13-AS05	32,944444	20,5138218	45,375067	0,0000002
AS09-AS07	-7,965556	-20,3961782	4,465067	0,4296197
AS10-AS07	6,565556	-5,8650671	18,996178	0,6572865
AS11-AS07	10,37	-2,0606227	22,800623	0,1519582
AS13-AS07	19,522222	7,0915995	31,952845	0,0005694
AS10-AS09	14,531111	2,1004884	26,961734	0,0141675
AS11-AS09	18,335556	5,9049329	30,766178	0,0012355
AS13-AS09	27,487778	15,0571551	39,9184	0,0000036
AS11-AS10	3,804444	-8,6261782	16,235067	0,9677286
AS13-AS10	12,956667	0,526044	25,387289	0,0368101
AS13-AS11	9,152222	-3,2784005	21,582845	0,268409

Dyes removal mechanisms

In the present study, tested dyes seem to interfere with all strain's fungal new cells formed at the tip of the hyphae and, often, with the entire mycelium (*Fig.9*). All fungal strains exhibited a second removal mechanism coupled with the enzymatic one on tested dyes. *Aspergillus sp.* AS02 and *A. iranicus* AS03 strains exhibited further biodegradation and less biosorption, *Cladosporium sp.* AS05 strain showed mainly biodegradation than biosorption, when fungal cells staining of *F. acuminatum* AS07 strain was observed increasing with the removal of the respective dyes from the medium without regained their original color, indicating strong biosorption and less biodegradation. Meanwhile, *H. carbonneanum* AS09 demonstrated mainly biodegradation and little biosorption activity. Finally, *Penicillium spp*. were further dyes biodegraders and seemed to be good accumulators.

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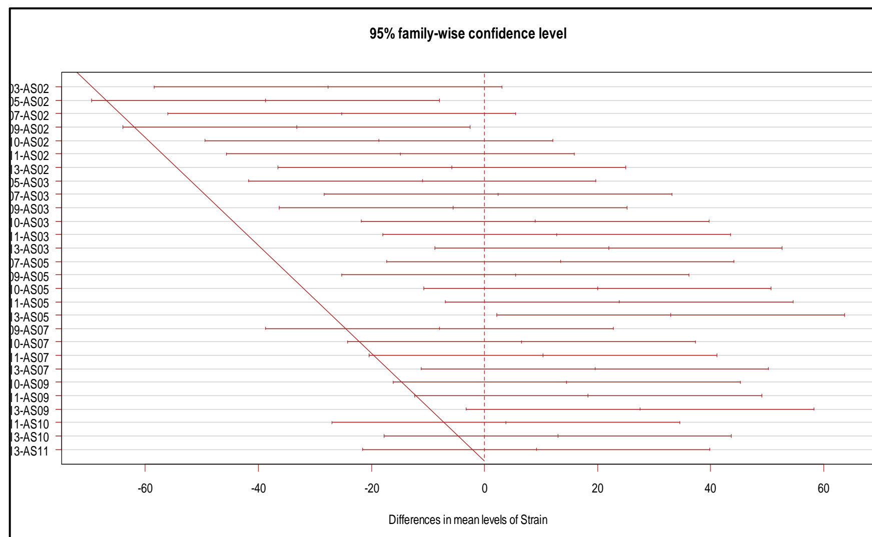

Figure 8. *Graphical presentation of pairwise comparisons between fungal strains by post-hoc TukeyHSD test for decolorization ability*

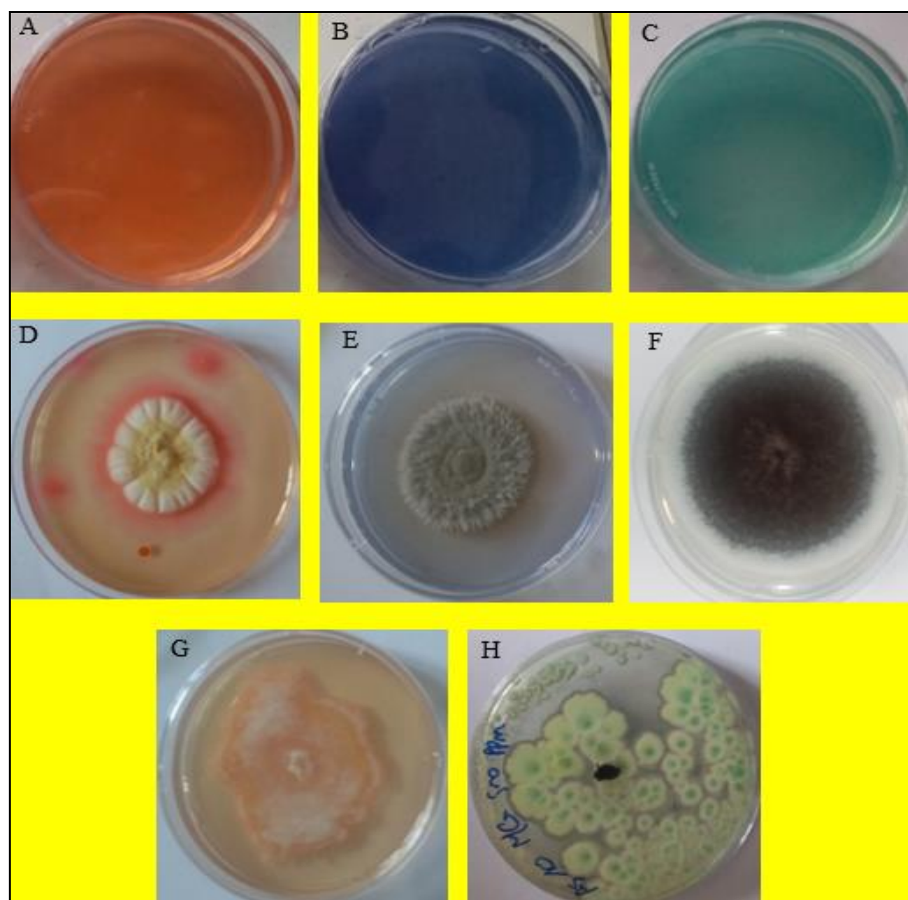

Figure 9. *Dyes decolorization mechanisms for microfungal strains of Ain Skhouna. A, B and C: negative controls 500 ppm of CR, BB and MG. D: adsorption of CR to the mycelium of A. iranicus strain AS03. E: biodegradation of BB by H. carbonneanum strain AS09. F: biodegradation of MG by Aspergillus sp. strain AS02. G: absorption of CR by F. acuminatum strain AS07. H: accumulation of MG by Penicillium sp. AS10 strain*

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Discussion

The aim of the present study was to evaluate the diversity of aquatic Micromycetes in the Ramsar registered Ain Skhouna wetland. Fungal diversity of any substrate depends on a large number of environmental factors such as pH, organic content, inorganic materials, concentration of dissolved oxygen, temperature and humidity level (Rangaswami and Bagyaraj, 2004; Yu et al., 2007; Medeiros et al., 2009). These factors affect not only the growth rate of fungi but can also trigger their development. Fungi can often tolerate one suboptimal factor if all others are almost optimal, but a combination of suboptimal factors can prevent fungal growth. For example, several fungi can grow at low pH (less than 4.0), and others can grow in anaerobic conditions, but few if any fungi can grow when low pH is combined with anaerobiosis (Deacon, 2006).

Measured water pH and salinity values fromsampling sites within Ain Skhouna wetland seem consistent with previous studies carried out by Daoud (1995) who gave estimated values of 7.35 for pH and 1.7g/L for salinity. They are therefore favourable for microfungi growth as they prefer acidic to neutral pH medium and tolerate a wide range of salinity (Grum-Grzhimaylo et al., 2016; Sharma et al., 2016). In contrast, high levels of inorganic ions, in particular bicarbonate (109 to 207.4 mg/L), magnesium (46 to 63.18 mg/L) and sulphate (397.9 to 459 mg/L) reported by Daoud (1995) could impact the diversity of Micromycetes. These parameters may vary, causing the formation of specific zones (Moubasher et al., 2018). The *in situ* measured temperatures could promote fungal growth, since most fungi are mesophilic, commonly growing within the range 10-40°C. Moreover, close environmental temperature changes recorded at site 1 and site 2 may not affects annual microfungal diversity. In contrast, the significant variations of temperature in sites 3 and 4 could influence it throughout the year. However, the dissolved oxygen concentrations here reported for all sites are suitable for microfungal development. We know that most filamentous fungi are aerobic or microaerophilic, but many yeast species and several mycelial fungi (e.g., *Fusarium oxysporium* and *Aspergillus fumigatus*) are facultative aerobes and can grow in the absence of oxygen by fermenting sugars (Tortora et al., 2004; Deacon, 2006).

The high fungal density of sediment may be due to its richness in nutrients and its capacity to trap Micromycetes drifting spores (Tsui and Hyde, 2003). In addition,due to their high plasticity and capacity to adopt various forms in unfavourable conditions, microscopic fungi are successful inhabitants of sediments (Palmer et al., 1997; Sun et al., 2005). Similarly, several authors (e.g., Findlay et al.,1990; Mille-Lindblom et al., 2006) demonstrated that in aquatic systems, fungal density differs greatly depending on substrate. However, the microfungal community described in this study consists most of aquatic–terrestrial Ascomycetes and only one genus of Mucoromycetes. In fact, many fungi are *in vitro* inculturable and produce hardly visible sexual structures (Tedersoo and Nilsson, 2016), so culture conditions does not allow all the present genera to be demonstrated. In addition, slow growing fungal species are overtaken by faster growing ones like *Mucoromycota* members (Wu et al., 2000). This may explain our results exemption of strains from other phylums like *Basidiomycota*. In general, this inventory seems consistent with the works of Kaushik and Hynes (1971), Kjøller and Struwe (1980) and Wu et al. (2013). They listed the fungal genera commonly found in wetlands including *Aspergillus*, *Alternaria, Cladosporium, Penicillium, Fusarium, Trichoderma* and aquatic hyphomycetes. Similar results were found by Doi et al. (2018) when studying water and sediments filamentous fungi density and diversity of the of Araçá bay (brakish water) in São Sebastião, São Paulo, Brazil, as well as Chikere and Azubuike (2014) from

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Ogala-Bonny river, Nigeria. Indeed, yeasts are ecologically flexible (Bogusławska-Wąs and Dąbrowski, 2001) and can be potentially found everywhere in freshwater and brackish water ecosystems (Moubasher et al., 2018) where dominant species are represented by *Candida, Rhodotorula* and *Cryptococcus* species (Hagler,2006). This may explain the abundance of *Candida sp.*in our samples along the year in both compartments which seems favoured by the local pH and calcium, magnesium and sulfate abundance ions as macroelements requirements (Marzluf, 1997; Rees and Stewart, 1997). Members of the fungal genera of *Penicillium*, *Aspergillus* and *Cladosporium*, which some strains were frequently isolated in this study, are cosmopolitan, well adapted to prevailing environmental conditions and colonizing a large substrates variety, especially in fresh water environment (El-Hissy et al., 1990; Tsui and Hyde, 2003; Hagler, 2006; Wu et al., 2013; Dirisu, 2015). Therefore, these ever-present taxa can be considered autochthonous (Botha, 2011). Otherwise, the remaining genera (*Fusarium*, *Mucor* and *Halobyssothecium*) are all either mesophilic or thermophilic organisms, known as field microfungi. Their moderate occurrence may be owing to antifungal substance productions by competing species, such as farnesol (Quorum Sensing Molecule) synthetized by *Candida sp.*, *Penicillium spp.* antibiotics and *Aspergillus spp.* toxins, inhibiting other fungal species growth (Deacon, 2006; Albuquerque and Casadevall, 2012; Sharma andRaju, 2013).

Concerning overall aquatic Micromycetes occurrence, Dayal and Tandon (1962) and El-Hissy et al. (1990)revealed that moderate temperature periods are favourable for aquatic Micromycetes growth, and their presence in water and sediments is affected by the external (terrestrial) climate change, as well as rainfall which significantly increase it both in land and aquatic ecosystems (de Araújo Pinto et al., 1992). Thus, constant occurrence of the same microfungal community in site 1 and 2 might be explained by the fact that these sites are permanently supplied with water from nearby springs (36°C) establishing a stable environment with little temperature and salinity fluctuations, which exerts little evolution pressure selection on the aquatic microfungi diverse forms, contrarily to site 3 and 4,where temperature changes throughout the seasons,being distant from the wetland's water original sources.

Molecular barcoding

Molecular barcoding using the ITS markers was not adequate enough to assign almost half of strains at the species level, despite it (ITS) have been shown useful in delineating many fungal species and is commonly used in identification and analysis of the molecular diversity of fungi (Baturo-Cieśniewska et al., 2020). This may be due to the lack of sufficient variability of the ITS region of the wells (Nilsson et al., 2012)which makes identification at species level more difficult(Seifert, 2009). In the literature, this feature was raisedespecially for some species-rich genera such as *Penicillium, Aspergillus* and *Cladosporium* (Skouboe et al., 1999; Schubert et al., 2007; Samson et al., 2014). This limitation associated with the analysis of ITS region to identify Ascomycota members was fully studied and discussed byBaturo-Cieśniewska et al.(2020), and consequently, for reliable species identification, it is recommended, in some cases, to combine ITS sequencing with fungal morphological features and/or other markers sequencing such as translation elongation factor 1-α(EEF1) (Meyer et al., 2019), nuclear large ribosomal subunit (LSU) (Liu et al., 2012), actin (*ACTB*) (Aveskamp et al., 2009), β-tubulin (*TUBB*) (Schoch et al., 2012), etc.

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Molecular barcoding assigned most strains of *Penicillium* to the sectionof the genusalthough the strain AS10 could be identified as *P. chrysogenum* since it was 100% identical to two species within *Penicillium chrysogenum sensu lato*, namely *P. chrysogenum* and *P. rubens* (Scott et al., 2004; Houbraken et al., 2011). Visagie et al. (2014), Houbraken et al. (2014) revealed high morphological confusion between species of this genus, which requires different culture media and incubated under different temperatures to be distinguished, in addition to sequencing preferably more than one molecular marker. Whereas, the existence of two distinct strains of *P. commune* (AS11 and AS12) might be an intraspecific diversity, which commonly occurs in some fungi (Baturo-Cieśniewska et al., 2020). Moreover, our literature prospection did not lead to any genetic sequencing data of *P. commune* from Algeria. So, we present here the first genetic information on this fungus. While for *Aspergillus* genus, AS02 strain was classified within *Aspergillus* section *Nigri* which includes some of the most important species for biotechnology (Samson, 2004) like *A.niger* species; very common in fungal communities and the largest industrially exploited (Meijer et al.,2011). Here too, to separate our strain *Aspergillus sp.* AS02 from its homologues another barcode is required due to themorphological similarity existing between members of the *Nigri* section (Varga et al., 2011), especially between *A. Niger* and *A. tubingensis*. Differently, the second sequenced strain of the genus, AS03 was successfully assigned as *Aspergillus iranicus*. This species was described for the first time in the *Aspergillus* section *Terrei* from hypersaline soil in Iran by Arzanlou et al. (2016), while in Algeria, in addition to the current study, it was isolated also from the subaqueous soil of the Djorf Torba dam located in the Wilaya of Bechar (Makhloufi et al., 2022), both from a non-saline substrate. However, strains AS05 and AS14 were identified as belonging to the *Cladosporium cladosporioides* complex which species-related are morphologically very similar and often only morphologically distinguished by careful observation and with detailed biometric data. Bensch et al. (2010) provided details on this complex. He analyzed more than 200 isolates based on DNA sequences of three markers: ITS, 1-α(EEF1) and partial actin, supplemented by phenotypic characters. He demonstrated that *Cladosporium cladosporioides* is an intricate complex containing several cryptic species, some of which remain unnamed. Furthermore, the description herein of *F. acuminatum* species (AS07) give more information on this fungus from Algeria where the first and unique report was made by Lazreg et al. (2013), as phytopathogen of Aleppo pine in northwestern Algeria. Finally, neither *H. carbonneanum* nor other species of the genus *Halobyssothecium* were previously described in Algeria. Hence, this is the first record of *Halobyssothecium* genus from the country.

Screening of fungal strains for laccases production

The experiment of laccases test was carried out using PDA medium supplemented with Bromophenol Blue in standard conditions of pH and temperature. Fungal laccases production depends on culture conditions (Rogalski et al., 1991; Schlosser et al., 1997). In fact, many compounds like Guaiacol (Coll et al., 1993),ABTS (Soden et al., 2016) and tannic acid (Mishra et al., 2011) have been tested by plate screen method on *Phanerochaete chrysosporium*; the “white rot model fungus”, *A. niger*, *A. terreus*, *Cladosporium oxysporum* and *Fusarium ventricosum* (Rani et al.,2014). Buddolla et al. (2008), Zhang et al. (2015), Kumar et al. (2018) and Senthivelan et al. (2019) documented that potential synthetic dyes removal of filamentous fungi is due to laccases in enzymatic remediation process. In the present study, high laccases activity exhibited by

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Aspergillus sp. AS02 and *Penicillium sp.* AS13 indicates the possible efficient azo and triphenylmethane dyes bioremediation.

Dyes decolorization assay

Mycelial growth

Studied strains seem tolerant to RC and BB since they have well mycelial growth rate. But, this doesn't necessarily match with dye decolorization process (Kang et al., 2014). Barrasa et al. (2014) and Kanagaraj et al. (2015) reported that dye mycoremediation process generates carbon and energy sources for fungal strain involved, depending on its ability to synthesize specific enzymes. This can be compared to promoted AS07 strain mycelial growth on CR and BB dyes. Whereas inhibition of fungal growth during the decolorization process might be due to the dye's toxicity, inhibiting enzymes production as well as cell growth (Singh, 2017) and, consequently, prevents dyes removal. In contrast, dos Santos Menezes et al. (2017) found out that lower rate growth of seven filamentous fungi on solid medium did not influence Methylene Blue dye degradation rate in liquid medium. Moreover, growth inhibition caused herein by MG dye against some fungal isolated strains confirms its antifungal activity, thus its used in aquaculture for this purpose (Kang et al., 2014).

Decolorized zone formation

Due to their chemical structures Azo and triphenylmethane dyes are hardly removed (Zhao et al.,2021; Shah et al., 2021), but through mycoremediation process, they are effectively decolorized by filamentous fungi, in particular by white rot fungi (Ramsay et al., 2005) and few non-lignolytic species, such as *Aspergillus, Penicillium, Fusarium* and *Cladosporium* (Rodríguez et al., 1996; Vijaykumar et al., 2006; Shahid et al., 2013; Almeida and Corso, 2014; Rani et al., 2014; Tehrani et al., 2014) whereas to our knowledge, the *Halobyssothecium* members' mycoremediation potential was never studied. In the present study, and by developing a light zone, we admitted that the fungus was able to decolorize the agar culture medium. Besides, comparison between mycelial growth (*Table 8*) and decolorized zone (*Table 9*) values indicates that they are proportional and decolorized zones diameters had a downward pattern with dyes concentration increasing. The results of the post-hoc Tukey test (*Table 10*) after Two-Way ANOVA for decolorized zone formation confirm and show that the decolorized zone formation is significantly influenced by dye concentration. Hence, as decolorization percentage is calculated on the basis of the diameter of the decolorized zone as given by the equation 2, we admit that the tested dyes concentration affects significantly on decolorization ability. This finding was frequently observed with white rot fungi species (Wang et al.,2017) where initial dye concentration increasing has a far-reaching effect on decolorization efficiency (Shabbir et al., 2018).

Percentage decolorization of dyes

It was possible to estimatedyes percentage of decolorization in solid medium using PVM method recently described by Shah et al. (2021) which allowed to compare our results.

According to the results of the decolorization assay, in particular those of decolorization percentage shown in *Figures 4*, *5*, *6* and *7*, *Aspergillus sp.* AS02 was the best efficient strain recorded in this study. Within the *Aspergillus Nigri* section, *A. niger* was widely studied for its decolorizing potential. It decolorized maximum CR at 74.07% and above 97% according

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to Shahid et al. (2013) and Asses et al. (2018), respectively. This species recorded 72.77% maximum decolorization of MG when studied by Rani et al. (2014). Another member of the section *Nigri*, namely *A. tubingensis* showed decolorization potential for pigmented molasses distillery wastewater by 44% (Watanabe et al., 2009) and 29.96% for CR (Thakor et al.,2022). Concerning *A. iranicus* AS03, no decolorization ability records are available, so our work is the first to focus on it, whereas the most well-known species of the section *Terrei*, *A. terreus* was already studied by Almeida and Corso (2014) where it displayed 98% decolorization of the azo dye Procion Red MX-5B in aqueous solution and, by Muthezhilane et al.(2008) reporting its potential (80%) against a wide range of synthetic dyes, including MG. However, there are few published studies on dyes decolorization ability for members of *Cladosporium cladosporioides* complex. Vijaykumar et al. (2006) reported that *C. cladosporioides* species decolorized five azo and triphenylmethane dyes, like Acid Blue 193 and Crystal Violet, both on solid and broth medium, while Muthezhilane et al. (2008) revealed a very low removal rate of dyes just like our C*ladosporium sp.*AS05 strain for CR and BB dyes. Concerning *F. acuminatum* AS07 strain, the unique work focused on this species was published by Yucel (2018) revealed its great removal ability (approximately 70%) against Methyl Orange (azo dye), meanwhile other species within the genus *Fusarium* and outside the *tricinctum* species complex, such as *F. oxysporum* and *F. solani,* showed dyes decolorization ability (Shahid et al., 2013; AI-Jawhari, 2015; Ting et al., 2021). Regarding dyes decolorization percentage values obtained with *H. carbonneanum* AS09, we suggest their use as a benchmark for upcoming studies since we didn't find any precedent similar work in the literature. *Penicillium sp.*AS10 strain was compared with its homologous within the section *Chrysogena* where *P. chrysogenum* still the most studied toward azo dyes. Bhattacharya et al. (2011) reported more than 50% decolorization rate for CR, while 89% removal/uptake of dye Reactive Black-5 was revealed by Erdal and Taskin (2016). However, Yang et al. (2011) reported 98.23% decolorization of MG by *Penicillium sp.* YW 01 strain genetically close to *P. chrysogenum* strain ATCC 10002. Whereas no scientific data was found on *P. commune* species neither on members of *Fasciculata* section, except *Penicillium sp.* YW 01 strain genetically similar to *P. commune* IBT 15141 strain. Finally, *Penicillium sp.* AS13 strain was very effective for dyes decolorization process against the three tested dyes at 3 concentrations, just like *Aspergillus sp.* AS02 as shown in *Figure 7* and confirmed by post-hoc test (TukeyHSD) results in *Table 13*. Comparable results were found by Hefnawy et al. (2017) for a strain of *P. canescens* homologous to *Penicillium sp.* AS13 within the *Canescenti* section, for the azo dye Direct Blue.

Dyes decolorization mechanisms

Interference of tested dyes at different concentrations observed with the new cells continually formed at the tip of the hyphae of studied fungal strains indicates primary adsorption before implication of different dyes removal mechanisms. Among these, enzymatic remediation process (degradation/decolorization), including laccases activity, seems the most promising (Knapp et al., 1995; Rani et al., 2014) than biosorption or accumulation. However, Singh (2017) revealed that concerted action of two mechanisms lead to greater remediation.

Remediation mechanisms shown here by *Aspergillus spp.* strains were observed elsewhere by Fu and Viraraghavan (2001), Almeida and Corso (2014) and Asses et al. (2018) for *A. niger* and *A. terreus* when removing azo dyes. According to Dionel etal. (2019), *C. cladosporioides* was able to efficiently remove Reactive Black B (RBB) azo dye by biosorption, unlike our *Cladosporium sp.* AS05 strain did. Biosorption mechanism was

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reported by Yucel (2018) on *F. acuminatum sp.* and by Seyis and Subasioglu (2008) for *Fusarium sp.* strain towards Methyl Orange. It's notable that either living or dead fungal biomass can be used as efficient synthetic dyes biosorbent (Srinivasan and Viraraghavan, 2010; Singh, 2017). Otherwise, we display here the first evidence about *H. carbonneanum* species dyes remediation mechanisms. Lastly, for *Penicillium spp*. strains, Yang et al. (2011) revealed that *Penicillium sp.* YW01 strain decolorized MG dye mainly by biodegradation.

On the light of these observations, percentage decolorization exhibited by fungal strains was more related to their production of laccases enzymes and then to their ability to absorbe and/or accumulate the respective dyes. Hence, screening for degradation enzymes production such as laccases is not always decisive for the selection of fungal strains intended for synthetic dyes remediation.

In brief, our results confirm specific synthetic dyes biodecolorization potential for several fungal strains belonging to different genera and their tolerance limits for dyes concentrations. However, mycoremediation were studied by several authors investigating influencing dye decolorization process parameters, such as pH (Praveen and Bhat, 2012), carbon and nitrogen sources (Rodríguez et al., 1994; Zhou et al.,2022), incubation temperature (Hefnawy et al., 2017) and inoculum size (Yang et al.,2011). It mainly focused on filamentous fungi, like *Aspergillus* and *Penicillium* strains (Tigini et al., 2009; Bano et al., 2018), so we suggest that some herein described strains, in particular AS02 and AS13, can be more effective after improving culture conditions, and therefore used as promising tool for dyes remediation from wastewater and industrial effluents containing synthetic dyes.

Conclusion

The Micromycetes described here represent Ain Skhouna wetland diversity in 2019. Our findings indicate that the sampling area exhibits favourable conditions of aquatic-terrestrial Ascomycetes development, such as constant water temperature. In fact, to highlight all mycoflora diversity in this ecosystem, the use of high-performance genetic techniques such as environmental DNA analysis must be carried out for further studies. In general, our results seem consistent with studies carried out worldwide in wetland areas. Molecular barcoding led to the characterization of 11 strains out of 14 isolated, 5 to the species level and 6 only to the section or complex within the correspondent genus. Consequently, the study provides the first genetic information for some fungi from Algeria, such as *P. commune* and *H. carbonneanum* and completes the little information available on *A. iranicus* and *F. acuminatum* species. Genetically identified strains were screened for their laccases production on PDA supplemented with Bromophenol Blue. Positive strains (8 strains) were investigated for their synthetic dyes decolorization ability on PDA using PVM. This technique indicated that some microfungal strains of Ain Skhouna wetland were able to decolorize synthetic dyes mainly through enzymatic mechanism. *Aspergillus sp.* AS02 and *Penicillium sp.* AS13 displayed the highest decolorization potential as promising fungal strains for dyes decolorization, while AS03, AS07, AS10 and AS11 strains exhibited moderate decolorization rates. In addition to fungal enzymatic action, biosorption and accumulation mechanisms can be useful for removing industrial effluents' synthetic dyes. We modestly suggest the use of obtained data as benchmark for local biodiversity monitoring and to recover fungal strains that could be economically viable for mycoremediation.

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APPENDIX

Table A1. *Monthly total count and community structure of aquatic microfungal taxa present in water samples from Ain Skhouna wetland during 2019*

Month	Individual fungal taxa CFU count average						
	Candida	**Penicillium**	**Aspergillus**	**Cladosporium**	**Fusarium**	**Mucor**	**Halobyssothecium**
January	280	42	40	8	12	0	0
February	277	86	55	29	17	0	4
March	366	98	67	44	21	7	15
April	457	109	81	45	27	15	23
May	493	103	84	53	26	31	11
June	536	120	74	41	20	24	8
July	481	70	41	16	5	11	1
August	392	51	39	0	2	8	0
September	412	66	57	17	0	14	0
October	463	72	61	23	0	21	5
November	610	73	57	31	7	26	10
December	477	24	17	19	7	11	7
Annual average	437	76,17	56,08	27,17	12	14	7

Table A2. *Monthly total count and community structure of aquatic microfungal taxa present in sediment samples from Ain Skhouna wetland during 2019*

Month	Individual fungal taxa CFU count average						
	Candida	**Penicillium**	**Aspergillus**	**Cladosporium**	**Fusarium**	**Mucor**	**Halobyssothecium**
January	413	51	35	15	0	0	0
February	422	75	54	36	8	0	7
March	479	130	73	58	29	14	8
April	525	127	79	86	41	17	13
May	569	175	93	93	38	20	24
June	596	151	84	47	28	21	27
July	506	75	65	19	7	8	11
August	431	57	43	3	0	3	2
September	530	82	56	15	7	29	0
October	610	110	62	45	10	17	9
November	602	106	60	37	5	11	7
December	509	50	29	26	9	5	0
Annual average	516	99,08	61,08	40	15,17	12,08	9

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