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Butachlor biodegradation potential of fungi isolated from submerged wood and surface water collected in Taal Lake, Philippines

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Taal Lake in the Philippines is a hotspot of diverse organisms but also faces a great threat from toxic agrochemical contaminants. Specifically, fungi are promising bioremediation agents to degrade harmful pollutants in the environment; hence the documentation and identification of these isolates are crucial. This study aims to determine the occurrence of fungi from submerged woods and surface waters in Taal Lake and assess their biodegradation potential against butachlor, a widely used pesticide in the Philippines. The isolated fungi were identified by using morphological and/or molecular methods, and the occurrence of each species was recorded. The ability of selected fungal isolates to degrade butachlor was also determined. A total of 28 morphospecies belonging to 20 fungal genera were recorded. Of these, eight fungal isolates grew on a chemically defined medium with up to 100 ppm butachlor. Two fungal isolates identified by molecular methods as *Neodeightonia subglobosa* IFM 63572 and *Sclerotium hydrophilum* IFM 63573 effectively utilized and potentially degraded butachlor as their sole carbon source as evident in the increased mycelial biomass (up to 0.449 g/L increase for *N. subglobosa* IFM 63572 and 0.214 g/L for *S. hydrophilum* IFM 63573) and decreased butachlor concentration (up to 94.68% reduction for *N. subglobosa* IFM 63572 and 89.64% for *S. hydrophilum* IFM 63573) after five days of incubation. Mycelial mat was more effective in degrading butachlor than mycelial balls. This study showed the presence of fungi from submerged woods and surface waters in Taal Lake and their potential application in the biodegradation of butachlor.

KEYWORDS

biodegradation, butachlor, freshwater-derived fungi, Taal Lake, *Neodeightonia subglobosa, Sclerotium hydrophilum*

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INTRODUCTION

Taal Lake is a notable freshwater habitat in the Philippines. This biome supports a diverse array of organisms ranging from endemic fauna (e.g., *Sardinella tawilis*, *Hydrophis semperi*) to different microorganisms that maintain an ecological balance in

this freshwater ecosystem. In spite of this, very few researches on the biodiversity of Taal Lake were conducted (Papa and Mamaril 2011). This is particularly true of the status of documenting its microorganisms, specifically fungi. Taal Lake also faces contamination of organic and inorganic substances from agricultural runoffs (Vista et al. 2006). The most persistent and toxic of these chemicals are the organochloride pesticides (Zacharia 2011). It was even suggested that the high chloride levels in Taal Lake waters and its sediment toxicity could be attributed to the presence and persistence of these chemicals in the lake (Hallare et al. 2009; Martinez and Galera 2011). Pesticides are known to have a negative impact on the environment and on health by affecting the cellular biochemical pathways and enzyme activities even at sublethal amounts (Zacharia 2011). Because fungi are among the best candidates for biodegradation, screening the local fungi in the lake would prove to be useful in the lake's rehabilitation against agrochemical pollutants.

Freshwater fungi contribute to the bulk of functional biodiversity in freshwater ecosystems (Geist 2011). They are also beneficial to the overall health of freshwater reservoirs as they function as decomposers, commensals, parasites, and predators (Wurzbacher et al. 2010; 2011). Furthermore, freshwater fungi can be tapped for their bioremediation potential against heavy metals (Iskandar et al. 2011) or complex xenobiotics (Junghanns et al. 2008).

This study aims to isolate, characterize, and identify fungi from submerged woods and surface waters collected from different sites in Taal Lake. Moreover, this study determines the ability of selected fungi to tolerate and potentially degrade butachlor [2-chloro-2',6'-diethyl-N-(butoxymethyl)acetanilide]. Hence this study can yield baseline information on fungi found in Taal Lake and, more important, show the potential of selected local fungi in the biodegradation of pesticides, i.e., butachlor. The occurrence of these fungi may help provide a solution for the rehabilitation of the lake against agrochemical runoff.

MATERIALS AND METHODS

Sample collection

Six areas within Taal Lake were chosen as sampling sites: (1) Talisay (14°04'19.2"N, 120°58'45.5"E), a local fish cage area; (2) Binintiang Malaki (14°2'13"N, 120°58'16"E), a coastal area in Taal Volcano; (3) Mataas na Kahoy (13°59'22.1"N, 121°05'00.3"E), a coastal residential area; (4) Alas-as (14°0'12"N, 120°59'42"E), a coastal area near Binintiang Munti; (5) Pansipit River (13°55′51.1″N, 120°56′59.2″E), a major tributary of Taal Lake, and (6) Pulang Bato (13°55'15.2"N, 120°57'55.5"E), a coastal area in San Nicolas. Water samples were taken randomly by using sterile, capped glass bottles submerged into the lake at approximately one foot in depth. The water samples were then transported in an icebox and processed within 24 hours. For woody substrata, 6 to 10 submerged wood debris and twigs were collected throughout the site. The samples were placed in large Ziploc plastic bags and transported inside an icebox.

Isolation and culture of fungi from surface waters

Four aliquots (12 mL) from each collected water samples were aseptically transferred to 15 mL sterile Falcon tubes and were then centrifuged at 500 rpm for 15 minutes by using a bench top centrifuge (Dynac, USA). Then about 10 mL of the top layer water was carefully decanted without disturbing the bottom portion. One mL of the remaining bottom layer was spread-plated on 1% Malt Extract Agar (MEA: 1 g malt extract, 15 g agar-agar, 1 L distilled water) supplemented with 0.5 g/L streptomycin sulfate. Antibiotic was added to inhibit bacteria. Four culture plates were prepared for each sampling site. The culture plates were incubated at room temperature (25–28°C) under normal laboratory lighting conditions and were observed for one week for fungal growth. Fungal colonies on the culture plates were subcultured on freshly prepared MEA until pure cultures were obtained on the basis of colony morphology and microscopic observation. A control plate was also prepared by exposing a MEA plate on the working area to ensure that the fungal isolates were not airborne contaminants.

Isolation and culture of fungi from submerged woods

The woody debris was initially cut into 3- to 4-inch segments and placed in clean disposable plastic containers lined with two layers of clean paper towels. Two to three woody substrates were placed in each moist chamber setup. Four moist chambers were prepared per sampling site. The moist chamber setups were then kept wet by pouring approximately 50 mL of freshwater collected from their respective sites. The moist chambers were incubated at room temperature (25–28°C) for four months and observed weekly for the presence of fruiting structures of freshwater fungi.

The fruiting structures were observed and aseptically removed from the substratum under a dissecting microscope (Olympus, Japan). The reproductive structures were then crushed and mounted with sterile distilled water on a clean, sterile glass slide. Under a compound microscope, spores were pipetted out and placed on petri dishes containing 1% MEA. The spores were observed daily for germination and hyphal growth. Agar blocks with fungal hyphae were cut out and placed on freshly prepared MEA. The cultures were incubated at room temperature and further subcultured on fresh MEA until pure cultures were obtained. For some ascoma and pycnidia, these were washed three times with sterile distilled water and surface-sterilized by dipping in 75% ethanol for five minutes. The surface-sterilized fruiting structures were allowed to air-dry aseptically for another five minutes for the solvent to evaporate. Following surface-sterilization, the fruiting structures were crushed and placed on freshly prepared MEA. Fungal colonies growing out of the fruiting structures were isolated and purified by using the agar block technique.

Characterization and identification of isolated fungi

Pure cultures of the fungal isolates were initially grown on MEA at room temperature for one week. Following incubation, hyphal and spore morphologies were determined from the isolated fungal cultures by using the Riddell slide method (Riddell 1950). Fruiting body structures of fungi isolated from woody substrata were also observed under a dissecting microscope. Various cultural and morphological characters were compared with online fungal databases for freshwater ascomycetes and teleomorphs (http://fungi.life.illinois.edu) and for tropical fungi (http://bcrc.firdi.org.tw/fungi) or with published morphological keys such as Seifert (1996) and Talaiekhozani and Ponraj (2015) to identify the isolated fungi. The occurrence of the fungi for each site was recorded.

Screening of selected fungi for growth on butachlor

Eight fast-growing and morphologically distinct fungal isolates were selected and screened for growth on butachlor. A chemically defined solid basal medium (CDSM) was prepared (Abd-Alrahman and Salem-Bekhit 2013). A stock solution of 5,000 ppm of butachlor (Sinochem, Philippines) was prepared by initially dissolving the butachlor with acetone. From the stock solution, six different concentrations of butachlor (20, 40, 50, 60, 80, and 100 ppm) were added to the autoclaved medium by using sterile 0.2 µm Whatman Syringe Filter (Whatman, UK). CDSM with the mineral salts only (blank), CDSM without butachlor (0 ppm) but supplemented with 0.1% sucrose, and CDSM with 50 ppm acetone (solvent) were used as controls. Then uniform circular agar blocks (11 mm in diameter) from

one-week old, pure fungal cultures were prepared by boring a flame-sterilized test tube mouth on the fungal colony edges and placed on the CDSM with butachlor and on control plates. The culture plates (in triplicates) were incubated for one week at room temperature (25–28°C). The diameter of each colony was measured for days 1, 3, and 5 post-incubation. The data were assessed by using two-way analysis of variance (ANOVA) and John Tukey's HSD (honest significant difference) test. The two best isolates with a significantly high capacity of growth on butachlor as the sole carbon source were used for the succeeding experiments.

Assay for the degradation of butachlor by using mycelial mats and mycelial balls

A chemically defined liquid basal medium (CDLM) was prepared by using the same formulation as described above but without agar. Chosen for inoculation on CDLM with 50 ppm butachlor were agar blocks from the fungal isolates *Neodeightonia subglobosa* IFM 63572 and *Sclerotium hydrophilum* IFM 63573 that exhibited best growth on CDSM with 100 ppm butachlor. The setups in triplicates were incubated at room temperature for 11 days for the formation of mycelial mats. For the preparation of setups with mycelial balls, the selected fungal isolates were initially inoculated on Potato Dextrose Broth and incubated in a shaker at 180 rpm and at temperatures ranging from 30°C to 35°C for four days. Following incubation, the mycelial balls were harvested by filtration, washed with sterile distilled water, and added on freshly prepared CDLM with 50 ppm butachlor. Three mycelial balls (about 0.03 g) were added in each culture setup. The inoculated culture media were kept under stationary condition for seven days at room temperature. In both setups, mycelial biomass was harvested on days 3, 5, 7, and 11, oven-dried at 100°C, and weighed until a constant dry weight was achieved.

The culture filtrates were also collected for butachlor analysis. Butachlor standard was first analyzed in High Performance Liquid Chromatography (Waters, USA) using a seven-point concentration (0, 6.25, 12.5, 25, 30, 40, and 50 ppm) calibration procedure to determine the retention time of butachlor. A linear regression was also computed, and the equation of the line was used to determine the relative concentration of butachlor in the subsequent analysis. Residual butachlor in the spent medium (filtrate) was initially detected by using a reversed phase C18 column (4.6 mm x 150 mm, 5 µm particle size; Phenomenex, USA). Separation was achieved by a flow rate of 1.0 mL/min with a mixture of distilled water and acetonitrile (40:60) mobile phase in an isocratic program. The injection volume was 20 µL. Data were integrated by Empower 3 software (Waters, USA) at wavelength of 215 nm. The extent of utilization and potential biodegradation of the pesticide was measured by detecting the residual butachlor in the spent medium and correlated with the mycelial dry weight as evidence of growth for the fungal isolates.

Confirmation of species identity of butachlor-degrading fungi by using molecular methods

The identity of the two most promising fungi was confirmed by sequence analysis of the intergenic spacer (ITS) region by using forward ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and reverse ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primer pair (White et al. 1990). The ITS region sequences of *N. subglobosa* and *S. hydrophilum* were deposited at the DNA Data Bank of Japan (DDBJ) with accession numbers LC177544 and LC177545, respectively. The sequences were analyzed and aligned by using ClustalW. A maximum likelihood tree was then constructed by using MEGA6 software, and the identity of the fungal isolates was established on the basis of the constructed phylogenetic tree. Pure cultures of *N. subglobosa* and *S. hydrophilum* were deposited at the Research Center for Pathogenic Fungi and Microbial Toxicoses (formerly, Institute for Food Microbiology, IFM), Chiba Medical Mycology Research Center in Japan with strain numbers IFM 63572 and IFM 63573, respectively.

RESULTS AND DISCUSSION

Occurrence of fungi in Taal Lake

A total of 28 fungal morphospecies were reported in this study: 12 morphospecies were derived from surface waters and 16 morphospecies came from submerged woods (tbl. 1). Of these, 26 morphospecies belonged to 20 fungal genera: *Acremonium*, *Amphisphaeria*, *Aspergillus*, *Aureobasidium*, *Campylocarpon*, *Cladophialophora*, *Cladosporium*, *Corollospora*, *Cylindrocarpon*, *Fusarium*, *Gliomastix*, *Lecythophora*, *Neodeightonia*, *Ophioceras*, *Penicillium*, *Pestalotiopsis*, *Phialemonium*, *Prosthecium*, *Sclerotium,* and *Trichocladium*. The recorded species were mostly Ascomycetes. Two fungal isolates out of the 28 morphospecies failed to produce spores in MEA after prolonged incubation and were noted as *mycelia sterilia* in the study. Since these two isolates differed in their colonial morphologies, they were considered as separate records.

Table 1: Occurrence of the different fungal taxa in the six sampling sites in Lake Taal. ALAS: Alas-as, TAL: Talisay, MATK: Mataas na Kahoy, BINM: Binintiang Malaki, PULB: Pulang Bato, PANR: Pansipit River.

Taxa	ALAS	TAL	MATK	BINM	PULB	PANR
Surface Water Fungi						
Acremonium crassum 4PULB05	-	-	-	-	+	-
Acremonium sp. 4MATK02	-	-	+	-	-	-
Aspergillus niger 3PULB03	-	+	+	-	+	+
Aspergillus flavus 6BINM02	-	-	-	+	-	-
Aspergillus fumigatus 8BINM03	-	-	-	+	-	-
Campylocarpon sp. 6FCA02	+	+	+	+	-	-
*Fusarium*sp.1 9MATK02	-	+	+	-	-	-
Neodeightonia subglobosa IFM 63572	-	+	-	-	+	-
Penicillium sp.1 5PULB05	-	-	-	-	+	-
Phialemonium sp.1 10BINM02	-	-	+	+	+	-
Mycelia sterilia 1 3ALA01	+	-	-	+	-	-
Mycelia sterilia 2 3BINM02	-	-	-	+	-	-
Submerged Wood Fungi						
Amphisphaeria sp. PANR03	-	-	-	-	-	+
Aureobasidium sp. MATK04	-	-	+	-	-	-
Cladophialophora subtilis PANR01	-	-	-	-	-	+
Cladosporium sp. BINM02	-	-	-	+	-	-
Corollospora sp. PANR05	-	-	-	-	-	+
Fusarium sp.2 BINM02-3	-	-	-	+	-	-
Fusarium sp.3 ALA03	+	-	-	-	-	-

Gliomastix sp. BINM02-2	-	-	-	+	-	+
Lecythophora sp. BINM02-1	-	-	-	+	-	-
Ophioceras sp. MATK05	-	-	+	-	-	-
Penicillium sp.2 MATK01	-	-	+	-	-	-
Pestalotiopsis sp. ALA04	+	-	-	-	-	-
Phialemonium sp.2 ALA03	+	-	-	-	-	-
Prosthecium sp. PULB04	-	-	-	-	+	-
Sclerotium hydrophilum IFM 63573	-	-	+	-	-	+
Trichocladium sp. ALA01	+	-	-	-	+	-

[a] (+) indicates the presence and (-) indicates the absence or nondetection of the fungal species in the sampling site.

A comparison of the six collection sites (tbl. 1) shows that Binintiang Malaki (BINM) has the highest number of taxa (10) followed by Mataas na Kahoy (MATK) (9 taxa), Pulang Bato (PULB) (7 taxa), Alas-as (ALAS) and Pansipit River (PANR) (6 taxa each), and Talisay (TAL) (4 taxa). The high number of taxa in Binintiang Malaki may be due to the abundance of littoral debris in this study site providing a more diverse microhabitat for the fungi, while the low number of taxa found in Talisay may be attributed to the scarcity of littoral debris as observed in this part of the lake.

Studies on fungi in Taal Lake are relatively few. In the study of Galan et al. (2013) on the different fungi recorded in waters of Taal Lake by using molecular methods, a total of 16 fungal species (both cultured and uncultured) were reported. In comparison, our study reports a comparatively lower number of species in surface freshwater alone (12 taxa). However, with the addition of the species reported on submerged woods, or the addition of 16 taxa, the total number of species in our study is higher than that of Galan et al. (2013). This would suggest that many "culturable" species remained unaccounted for in Taal Lake and perhaps can be even found in substrates other than surface waters, e.g., in sediments, bottom water, macrophytes, to name a few, that can serve as microhabitats for these fungi. In our study, some of the taxa reported from surface waters and submerged wood belong to the fungal genera *Acremonium*, *Cladosporium*, *Corollospora*, *Cylindrocarpon*, *Fusarium*, *Penicillium, Phialemonium*, *Prosthecium,* and *Trichocladium* (tbl. 1). These taxa are known major fungal constituents of freshwater habitats (Shearer et al. 2007; Wurzbacher et al. 2011) with two genera, *Corollospora* and *Acremonium*, reported as common in woody substrates (Josephine et al. 2011). The genus *Aspergillus*, also reported in our study, is considered as an aeroaquatic species in lotic freshwater habitats (Prasad et al. 2009). *Aspergillus* is most commonly associated with polluted water systems where it decomposes plant materials such as cellulose (Bandh et al. 2011). Interestingly, we also recorded two fungal species known as plant pathogens. *N. subglobosa* is reported as a common pathogen and saprobe in woody plants (Trakunyingcharoen et al. 2015), while *S. hydrophilum* has been known to be associated with aquatic and semiaquatic plants (Xu et al. 2010). In our study, these two fungi grew best on CDSM with butachlor as sole carbon source (fig. 1; fig. 2) and hence were used as the target organisms for the biodegradation of butachlor. ITS region sequence analysis further confirmed the identities of *Neodeightonia subglobosa* IFM 63572 with a bootstrap probability value of 93% and of *Sclerotium hydrophilum* IFM 63573 with a bootstrap probability value of 100% (fig. 6). *N. subglobosa* is under the family *Botryosphaeriaceae* and is closely related to the genus *Diplodia* (Dissanayake et al. 2016), while *S. hydrophilum* is the synonym of *Ceratorhiza hydrophila* and is closely related to the genus *Ceratobasidium* (Xu et al. 2010).

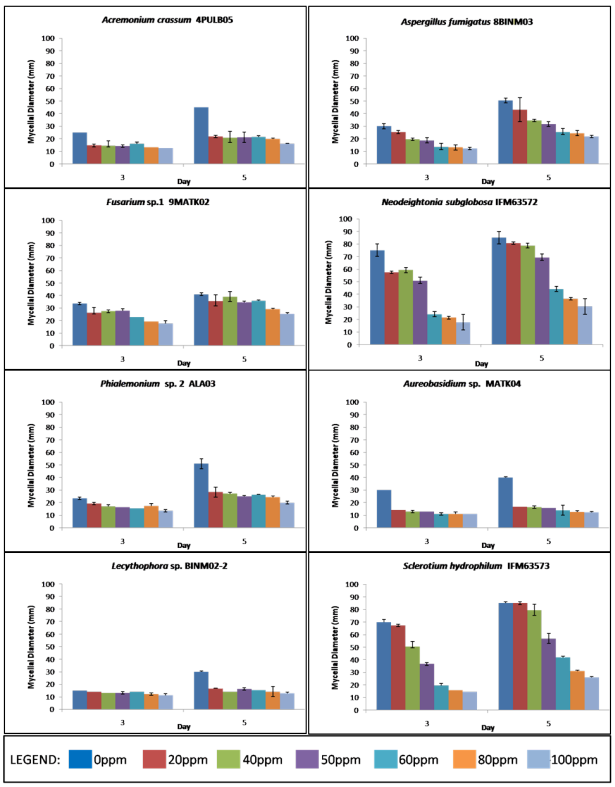

Figure 1: Mean mycelial diameter (in mm) of the eight fungal isolates inoculated on CDSM with different concentrations of butachlor after three and five days of incubation. Error bars indicate standard deviation.

Screening of fungi for degradation of butachlor

Butachlor is an organochloride herbicide that is widely used in the Philippines against weeds. It is also considered as a major agrochemical contaminant in Philippine soils and waters. Previous studies also showed the negative effects of this herbicide. For example, butachlor negatively affected the growth of beneficial microorganisms such as the nitrogen-fixing cyanobacteria in paddy fields (Zargar and Dar 1990). It is also highly toxic to several aquatic organisms and known to interfere with the biochemical process of the neurons (Tilak et al. 2007). The presence of pesticides and other residual agrochemical components had been noted in Taal Lake (Vista et al. 2006; Hallare et al. 2009). These agricultural runoffs can find their way to fishes and other aquatic animals in the area and can also adversely affect humans if such fishes and aquatic animals are consumed.

Eight fungal isolates, four isolated from surface water (*Acremonium crassum* 4PULB05, *Aspergillus fumigatus* 8BINM03, *Fusarium* sp.1 9MATK02, and *Neodeightonia subglobosa* IFM 63572) and the other four isolated from submerged wood (*Aureobasidium* sp. MATK04, *Lecythophora* sp. BINM02-1, *Phialemonium* sp.2 ALA03, and *Sclerotium hydrophilum* IFM 63573), were grown on CDSM with 0 to 100 ppm butachlor (fig. 1). All eight fungal isolates grew on CDSM with butachlor at up to 100 ppm for seven days (fig. 2), indicating their ability to utilize butachlor as sole carbon source. This also shows the capability of the eight fungal isolates to degrade butachlor even up to 100 ppm concentration. However, only thin vegetative mycelia and minimal production of aerial mycelia were observed on all eight fungal isolates. The highest colony diameter at day 3 and 5 was recorded for *N. subglobosa* IFM 63572 and *S. hydrophilum* IFM 63573 (fig. 1). Both fungal

isolates also covered the entire agar culture plate with its mycelia after five days of incubation. Based on their growth on CDSM, a 50 ppm butachlor concentration was used in the assay with a liquid culture medium as similarly done by Abd-Alrahman and Salem-Bekhit (2013).

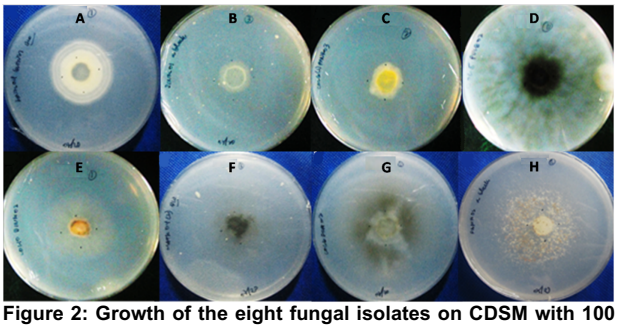

Figure 2: Growth of the eight fungal isolates on CDSM with 100 ppm butachlor after seven days of incubation: (A) *Acremonium crassum* 4PULB05, (B) *Aspergillus fumigatus* 8BINM03, (C) *Fusarium* sp.1 9MATK02, (D) *Neodeightonia subglobosa* IFM 63572, (E) *Phialemonium* sp.2 ALA03, (F) *Aureobasidium* sp. MATK04, (G) *Lecythophora* sp. BINM02-1, and (H) *Sclerotium hydrophilum* IFM 63573.

Fungi are known to be capable of tolerating and even degrading chemical contaminants (Mendoza et al. 2010; Torres et al. 2011; Torres and dela Cruz 2013). *Aspergillus* and *Fusarium* species were reported to be herbicide-tolerant fungi (Torra-Reventos et al. 2004; Abdel-Megeed et al. 2013). In our study, we further corroborated this finding. Two fungal isolates identified as *Aspergillus fumigatus* 8BINM03 and *Fusarium* sp. 1 9MATK02 grew on CDSM with 100 ppm butachlor (fig. 1). These two fungal genera are widely dispersed in the environment and have developed tolerance to most pollutants particularly in wastewater (Grover et al. 2007). In addition to freshwater fungi, wood-rotting species were reported capable of tolerating high levels of pesticide pollution by producing extracellular enzymes and increasing mycelial extension (Roca et al. 2009). As evidently shown in our study, the two fungal isolates, *N. subglobosa* IFM 63572 and *S. hydrophilum* IFM 63573, exhibited the highest mycelial diameter even in the presence of 100 ppm butachlor (fig. 2). *N. subglobosa* was previously reported as a wood-rotting fungus, although in our study we isolated this fungus from surface waters. Roca et al. (2009) hypothesized that the rapid mycelial growth of fungi in chemically polluted and nutrient-limited habitats facilitates the exploration of the fungi for possible alternative carbon sources.

The tolerance of a certain fungus to a specific chemical pollutant can be a basis for exploiting the fungus for application in environmental cleanup or bioremediation. In our study, we tested *N. subglobosa* IFM 63572 and *S. hydrophilum* IFM 63573 for their ability to potentially degrade butachlor. The High Performance Liquid Chromatography (HPLC) profile of the butachlor standard was then determined and observed to have a retention time of 6.354 minutes (fig. 3). The results showed a continuous decrease in the concentration of butachlor present in the spent medium as the incubation period progressed (fig. 4; fig. 5). Evidently the two fungal isolates utilized and reduced the amount of butachlor present in the assay medium. Using the fungus *Mucor*, Chen and Wu (1978) observed that the effective release of degradative enzymes facilitates the utilization of the pesticide by breaking down the complex chemical into easily metabolized compounds. Our study also showed the continuous decrease in the amount of butachlor (up to 94.68% reduction for *N. subglobosa* IFM 63572 and 89.64% for *S. hydrophilum* IFM 63573) in the spent medium and the consequent increase in the mycelial dry weight (fig. 5) indicating potential degradation and utilization of butachlor by the two fungi. Growth conditions can be a factor why butachlor is still present in the culture medium until the last day of incubation. For instance, according to Torra-Reventos et al. (2004), degradation of butachlor by a specific strain of *Aspergillus niger* can be optimally achieved if sucrose is present as the primary carbon source. Since our study is limited only to the use of CDSM with butachlor as the sole carbon source, a slower fungal metabolism possibly occurred, and therefore a complete reduction in the butachlor was not observed until the last day of incubation. However, what is highly possible is that a 100% reduction could have been observed if the incubation period was extended. Enzymatic requirements can also be a factor as indicated by Agrawal et al. (2015). In their study, the enzyme AKR17A1, a novel enzyme from *Anabaena* sp. PCC7120, was found to degrade butachlor only in the presence of the enzyme cofactor NADPH. Hence, during the period of maximal growth of the fungi (day 5 onward for mycelial mat and day 3 onward for mycelial balls), a potentially slower degradation of butachlor possibly occurred in the culture medium.

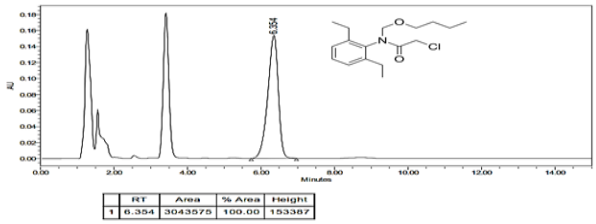

Figure 3: HPLC chromatogram of the butachlor standard used in the analysis. The typical parabolic peak with the retention time of about 6.3 minutes indicates the presence of the butachlor.

The two setups used in our study were the mycelial mat and the mycelial balls. Mycelial mat is the natural growth form of most filamentous fungi. However, this form may not be suitable for the actual bioremediation process because of its limited nature of growing only on the top of the substrate and being easily destroyed by moving currents. Meanwhile, mycelial balls are the growth form of filamentous fungi which can be induced by using a rotary shaking medium. If used *in situ*, mycelial balls can be easily applied in the environment as compared to mycelial mat.

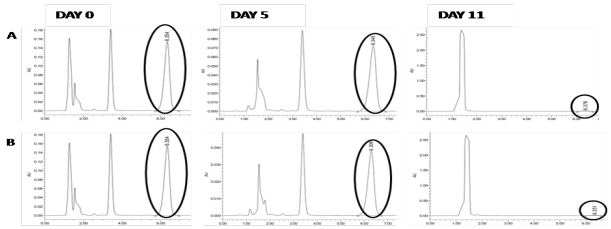

Figure 4: Chromatograms for mycelial mat showing gradual decrease in butachlor from day 0, day 5, and day 11 as indicated by the area under the peak of the curve: (A) *N. subglobosa* IFM 63572 and (B) *S. hydrophilum* IFM 63573.

For the mycelial mat setup, the decrease in butachlor was evident until the last day of incubation with 94.68% reduction for *N. subglobosa* IFM 63572 and 89.64% reduction for *S. hydrophilum* IFM 63573. A significant decrease (p value = 0.03403, $\alpha = 0.05$) was observed until day 5. Our results also showed that mycelial mat tended to reduce almost completely the amount of butachlor in the medium as compared to the response of mycelial balls (fig. 5). This can be due to the mycelial mats having a greater surface area exposed to the medium, thereby facilitating a more effective enzyme release and hastening the breakdown of butachlor present in the medium. By contrast, the mycelial balls setup exhibited significant decrease (p value = 0.03904, $\alpha = 0.05$) only until day 3. The limitations of growth of mycelial balls can be explained

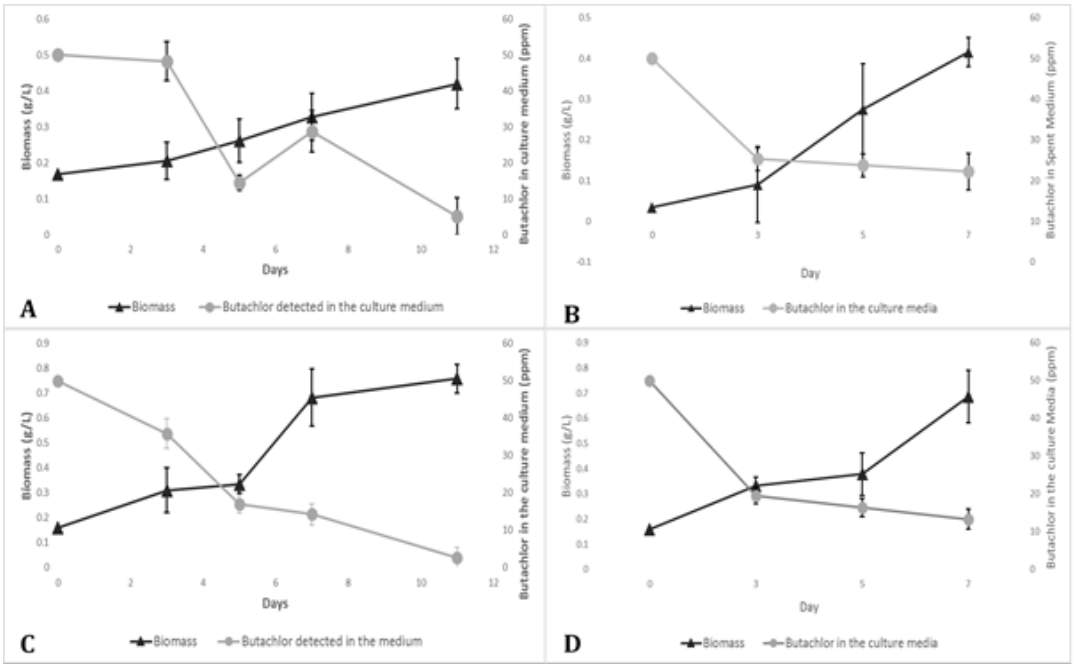

Figure 5: Correlation between the biomass (g/L) of the two isolates mycelial mat and the residual butachlor in the spent medium (in ppm) over a period of time: (A-B) *S. hydrophilum* IFM 63573 and (C-D) *N. subglobosa* IFM 63572. Error bars indicate standard deviation.

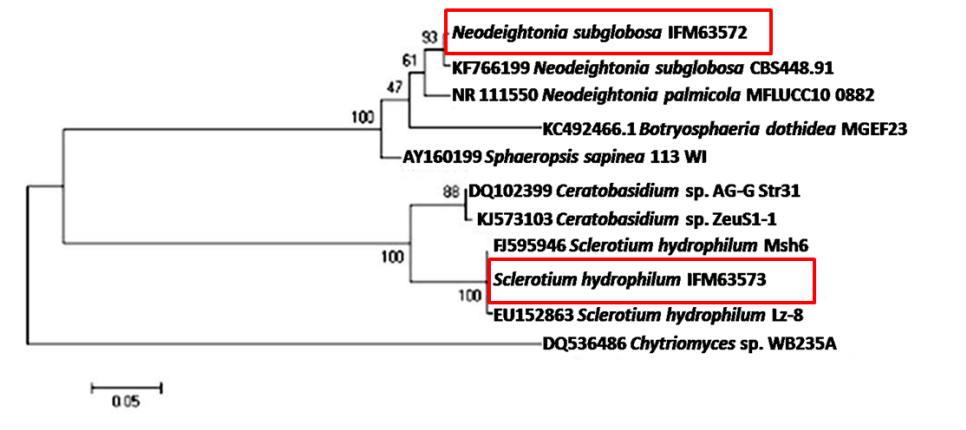

Figure 6: Phylogenetic tree confirming the identity of *S. hydrophilum* IFM 63573 and *N. subglobosa* IFM 63572. *Chytriomyces* sp. was used as an outgroup.

by the diffusion-limitation of oxygen and nutrients governed by mycelial growth kinetics (Chiu and Zajic 1976). Reductions of 73.4% for *N. subglobosa* IFM 63572 and 55.6% for *S. hydrophilum* IFM 63573 were observed until the last day of incubation. When the amounts of butachlor in the spent medium for the two setups at day 3 were compared, a greater reduction (60.94% for *N. subglobosa* IFM 63572 and 49.42% for *S. hydrophilum* IFM 63573) was observed in mycelial balls. However, the total reduction until the last day of incubation is greater in the mycelial mat setups. The observed effective utilization of butachlor occurred after 5 days in mycelial mats and after 3 days in mycelial balls. The initial lag period may indicate the metabolic adjustment of the fungi in response to the new carbon source (butachlor).

When the relationship between the mycelial dry weight of the fungi and the residual butachlor present in the spent medium (fig. 5) was assessed, an inverse relationship was noted. This means that as the amount of butachlor detected in the spent medium decreased, the mycelial dry weight increased. However, for the mycelial mat, only during day 5 did the fungi actually begin utilizing well the butachlor in the culture medium. As observed, the mycelial dry weight at day 5 started to increase relative to the reduction in the amount of butachlor in the spent medium. The net increase in the mycelial biomass for this setup until the last day of incubation was 0.449 g/L for *N. subglobosa* IFM 63572 and 0.214 g/L for *S. hydrophilum* IFM 63573. For mycelial balls, the net increase in mycelial biomass was comparatively lower, i.e., 0.354 g/L for *N. subglobosa* IFM 63572 and 0.084 g/L for *S. hydrophilum* IFM 63573.

Overall, our study implies that Taal Lake is a freshwater habitat which caters to diverse and unique assemblages of microorganisms, including freshwater fungi. However, our study covered only a small portion of the fungal biota found in Taal Lake. Hence we recommend an assessment of fungal diversity in other substrates from Taal Lake where fungi can thrive. This would result in a more nearly complete enumeration of the fungal species present in the lake. Our study also implies that some fungi have a potential application in pesticide biodegradation in freshwater habitats.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

Mark B. Carascal, Marlo Jose del Rosario, Kin Israel R. Notarte and Thomas Edison E. dela Cruz did the sampling, planning, experimentations, data analysis, and/or manuscript writing. Fahrul Huyop assisted in the conduct of the butachlor degradation assay, while Takashi Yaguchi assisted in the molecular identification of the fungal isolates.

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