ISOLATION AND MORPHOLOGICAL CHARACTERIZATION OF FILAMENTOUS FUNGI PRESENT IN HEXACHLOROCYCLOHEXANE CONTAMINATED SOILS

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Abstract: Persistent organic pollutants cause a myriad of ecosystem perturbances with long lasting consequences for their resident animal, plant and microbial communities. Structural shifts in biodiversity and changes in population dynamics are inextricably tied to the introduction of anthropogenic pollutants. Sequentially, contaminated ecosystems represent an interesting field of inquiry as to the lines of succession following the introduction and continued existence of such toxic compounds. Soil microbial communities in particular have been described as the first responders to both trace and abundant amounts of pollutants. Hexachlorocyclohexane (HCH) is one such persistent organochloric pollutant, historically used as a wide spectrum insecticide and acaricide in agriculture and medicine. HCH's long soil half-life has ensured it remains a continuous pernicious problem for areas where extensive HCH manufacture and utilization have occurred. The Skopje valley is one such area, where within the boundaries of the factory OHIS, approximately 30 million kilograms of technical grade HCH waste has been disposed of in two landfills. These landfills act as active loci of HCH contamination, allowing slow and steady infiltration onto surrounding areas, including fertile farm fields, human communities and a small riparian biotope. In order to study the microbial inhabitants of HCH contaminated soils, samples were acquired from surrounding areas and within the official boundaries of OHIS. These samples served as initial matrices for the isolation of filamentous fungal strains with the end goal of surveying the various distinct qualitative morphological adaptations present among these communities. Among the 35 morphologically unique strains, the majority showed hirsute morphology (74%), septate hyphae (71%) and belonged to the phylum Ascomycota (88%). The findings of this study suggest that similar physiognomic strains occupy similar functions in contaminated ecosystems. Studying these microbial inhabitants and their various adaptations may yield novel insights into potential bioremediation avenues for managing HCH and other organochloric contaminants. Thus the goal of the presented study is to evaluate the fungal microbial communities present in the HCH contaminated soils near OHIS, North Macedonia. By determining certain trends of occurrences, these communities may then further serve as pools for the selection and development of novel bioremediation agents for the betterment of contaminated areas across the whole of North Macedonia.These isolates could contribute for the improvement of the wider market for soil remediation products and could also be utilized to create a clean enviorment for all of us. Isolates can participate in the production of new Macedonian product used for bioremediation processes.
Keywords: Bioremediation, Soil, HCH, Ascomycota

1. INTRODUCTION

The historic use of Hexachlorocyclohexane (HCH) across Europe for the larger part of the 20th century has resulted in over 2 million tonnes of HCH waste allocated across several hotspots on mainland Europe (Vijgen et al. 2019). These areas act as key loci for cross contamination of HCH residues onto subterranean and surface water systems (Di et al. 2018), accompanied by mass scale bioaccumulation and biomagnification across members of associated food chains (Kolaříková et al. 2012). According to Vijgen et al. (2010), 35 thousand tonnes of HCH waste is present within the borders of North Macedonia, equating to about 2% of the estimated total amount of HCH waste present in Europe. The entirety of these 35 thousand tonnes is concentrated in the Skopje valley, owing to the historic mass production of HCH by the factory OHIS, making the factory and the surrounding areas act as the number one hotpot in industrial contamination even after the ceased production of HCH (Stafilov et al. 2009; Stafilov et Sajn 2019).

The entirety of this toxic HCH load is allocated between three disproportionate landfills, two of which are located near the factory OHIS with the third one presumed to be located near a monastery in Dračevo, Skopje. These landfills are frequently noted as public health and environmental hazards of the highest order. (Kosteska et Gjorgjev 2020).

From these landfills, HCH isomers slowly infiltrate associated ecosystems and human communities, where they exert their pernicious effects. A notable study in 2007 by Krstevska-Konstantinova et al. found that serum presence of organocholride pesticides, particularly HCH, was associated with prepubescent breast budding in girls from the

Skopje valley. Similar studies have shown that HCH waste regularly integrates into the public health of nearby human settlements, causing various long term issues including an increased incidence of Parkinson's disease (Richardson et al. 2011), liver disease (Kutz et al. 1991), endocrine disruptions as well as increased oxidative stress and DNA damage(Rubini et al. 2020). Because of these effects, HCH isomers are identified as biohazards for immediate remediation. However, very little focus is drawn to the ecotoxicity and the induced adaptive ecosystem responses of these HCH isomers. Bioaccumulation and bioconcentration studies have shown that this compound and its isomers are highly lipophilic to a degree that their incorporation into the animal and vegetative communities of affected areas is unquestionable (Viswanathan et al. 1988; Monterroso et al. 2002; Kolaříková et al. 2012). Yet the exact qualitative responses are seldom addressed, with a notable ignorance in the case of microbial communities.

The particular aim of the presented paper is to evaluate those pathophysiological effects among the native fungal microbiota of HCH contaminated soils. Fungi occupy key roles in every terrestrial ecosystem they inhabit. The range of metabolic functions and positions they engage in stretches from nitrogen and carbon refluctuation to mineral mobility control and, most notably, xenobiotic bioprocessing. In fact, terrestrial hyphal fungi have been known to readily respond to the presence of recalcitrant molecules, including HCH (Winquist 2014; Maqbool et al. 2016; Russo et al. 2019a). Sainz et al. (2010) in particular found a significant relationship between the number of arbuscular mycorhizzal propagules of native fungi and the degree of HCH contamination, affirming a conclusion that such fungi are negatively impacted by present HCH isomers. Similarly, Ceci et al. (2019) found that certain saprotrophic fungi coalesce in contaminated soils, showing that qualitatively similar strains exhibit similar behaviour under ecosystemic stress. It is by no stretch of the imagination that fungal bioremediation agents are most often found as members of these reactive microbial consortia (Quintero et al. 2008; Ceci et al. 2015).

2. MATERIALS AND METHODS

Sampling

Sampling was carried out by extracting a singular vertical transect (15 cm depth) of soil. Approximately 500 grams of each soil were allocated in sterile polyethylene bags which were then sealed in sterile brown paper bags as to minimize ultraviolet interference in the sample's microbiota and reduce the risk of cross-contamination. Soil samples were acquired from OHIS and the surrounding area (Senanayake et al. 2020), including samples from soils in immediate vicinity to the two official landfills, termed the large and small landfill respectively (Figure 1), as well as from the third illicit landfill near Dračevo, Skopie. Recorded coordinates are shown in table 1. The samples were transported in a polypropylene container under a constant temperature of 4 ^{o}C (± 4 ^{o}C) to the laboratory where isolation would be carried out within 24 hours of reception of the samples.

Isolation and cultivation media

In order to isolate soil dwelling fungal strains, a modified Minimal Salt Medium (MSM) medium was utilized (Hartmans et al. 1992; Coleman et al. 2002). The base medium contained 0.5 g * L^{-1} KH2PO4, 0.5 g * L^{-1} K2HPO4, 0.5 g * L^{-1} MgSO4x7H2O, 0.1 g * L^{-1} CaCl2, 0.2 g * L^{-1} NaCl, 0.01 g * L^{-1} FeSO4x7H2O, 0.01 g * L^{-1} MnSO4x7H2O and 1 g * L^{-1} NH4NO3 with adjusted pH=7. This base mineral composition served as an ideal liquid medium for transitioning soil dwelling microorganisms from an initial oligotrophic matrix to a nutrient richer medium for later isolation and characterization. In order to facilitate fungal growth in MSM, glucose was added to MSM with a final concentration of 10 g * L^{-1}. Additionally, in order to better simulate their native environment, aliquots of an acetone based HCH solution were added in order to continuously maintain the presence of the pollutant so as not to cause potential changes in strain characteristics. All media used were autoclaved at 121 ^{o}C for 15 mins and allowed to cool to room temperature before use. The HCH standard solution was prepared by dissolving 1 g of an analytic grade HCH mixture in 100 ml of pure analytic grade acetone, equating to a 10 000 ppm standard solution. The HCH mixture used was comprised of 79% (w/w) alpha-HCH, 15% beta-HCH, 5% gamma-HCH and 1 % delta-HCH. This mixture was purposefully selected as it best simulated the HCH waste which most likely permeates the soils sampled. All cultural and morphological assessments were carried out on solid plates of Sabouraud Dextrose Agar (SDA, Biolife), containinig 5 g * L^{-1} pancreatic digest of casein, 5 g * L^{-1} peptic digest of meat, 40 g * L^{-1} glucose and 15 g * L^{-1} agar. Sterility was assured by autoclave at 121 ^{o}C for 15 mins, allowing the medium to cool to 50 ^{o}C before plating. SDA represents a standard nonselective medium for fungi from clinical and environmental samples (Campbell et al. 2013).

Figure 1. Physical vicinity of the large and small landfill in OHIS. Image property of the Ministry of Environment and Physical Planning of North Macedonia (moepp.gov.mk).

Isolation of filamentous fungi

Initial suspensions were prepared by dilution of 100 grams of soil in 100 ml of sterile MSM (10% glucose and 10 ppm HCH fortified). The slurry mixture was allowed to stabilize for approximately 60 minutes, after which 1 ml aliquots from each sample were plated on SDA. The plates were incubated at 25 ºC (± 0.5 ºC) for 5 days. The Erlenmeyer flasks were set onto an agitation platform set to 120 Rotations per Minute (RPM). The 19 flasks were allowed to incubate as shake flask cultures at 25 ºC (± 1 ºC), 120 RPM, for 5 days. The agitation platform was covered to exclude the interference of light.

After 120 hours, the flasks were set aside and allowed to stabilize before 1 ml aliquots were plated on SDA. The plates were incubated identically as before. From each flask culture, the upper liquid phase containing viable fungal propagules was carefully decanted in sterile centrifuge cuvettes. The soil sediment was decontaminated before elimination. Centrifugation of the liquid phase of each separate sample was carried out at 4000 RPM for 15 minutes. The supernatant was decanted, decontaminated and eliminated appropriately. The sediment containing all viable fungal material was resuspended in sterile MSM and centrifuged again in an identical manner. The sediment was resuspended once more in sterile MSM in order to ensure thorough elimination of HCH residues and interfering metabolites which might inhibit the competing strains of the sample. After the second biomass cleansing, the sediment was suspended in sterile MSM and transferred in Erlenmayer flasks containing sterile MSM (10% glucose and 50 ppm HCH fortified). The higher concentration of HCH served as an artificial selection parameter, allowing only 50 ppm tolerant fungal strains to thrive. The 19 flasks were incubated at 25 ºC (± 1 ºC), 120 RPM, for 5 days with minimal light interference.

The isolation process was repeated identically two more times, raising the HCH concentration from 50 to 100 and then from 100 to 200 ppm. After the final 120 hours of incubation at 200 ppm HCH, isolation was carried out directly from the flask cultures, and the mixed cultures were then decontaminated and discarded appropriately.

Utilizing an inoculation needle, subsampling was carried out on all distinct homogenous growths present on the SDA plates after 5 days of incubation. Inoculi from these points were transferred onto sterile SDA plates and allowed to incubate for five days at 25 ºC (± 0.5 ºC), after which they were revaluated for cultural purity. Impure or mixed cultures were subject to further subcultivation until pure cultures were achieved.

Cultural and morphological characterization

Pure axenic fungal cultures grown on SDA plates were evaluated thoroughly for observable cultural characteristics (Campbell et al. 2013). The qualitative markers noted during this evaluation included: colour, smoothness, hirsuteness and visible differentiation of the central and peripheral regions of the colony in question (Senanayake et al. 2020).

Native microscopy slides from the same cultures were prepared using lactophenol (Campbell et al. 2013) and microscopic details obtained served to place each culture in one of the following groupings (Senanayake et al. 2020): septate / nonseptate (coenocytic) and possessing ascospores / basidiospores / asexual conidiospores. In the

latter group case, the presence of conidiospores was only noted in utter absence of sexual ascospores and basidiospores.

Attained data served to compile unique profiles for each potentially unique culture. The profiles were then compared with each other, before forming a final list of assuredly unique filamentous strain. Only those strains whose morphological and cultural characteristics were unique (Cappuccino and Sherman 2002) to the sample they were isolated from were deemed unique fungal strains present in the respective soil.

Table No. 1. Geolocation data of sampling points

Sample	Matrix	Geolocation	Location
1	Pure soil	41°57'46.6"N 21°29'00.3"E	Large landfill, OHIS
2	Soil w/ suspect mycelia	41°57'45.7"N 21°28'59.4"E	Large landfill, OHIS
3	Pure soil	41°57'42.9"N 21°28'59.6"E	Small landfill, OHIS
4	Soil w/ suspect mycelia	41°57'42.4"N 21°28'57.7"E	Small landfill, OHIS
5	Pure soil	41°57'36.1"N 21°29'23.6"E	OHIS
6	Pure soil	41°57'39"N, 21°29'10"E	OHIS
7	Pure soil	41°57'38"N, 21°29'05"E	OHIS
8	Pure soil	41°57'17"N, 21°31'49"E	Farmlands near OHIS
9	Pure soil	41°57'19"N, 21°31'47"E	Farmlands near OHIS
10	Pure soil	41°57'33.5"N 21°29'43.6"E	Near OHIS
11	Pure soil	41°57'44.9"N 21°29'19.6"E	Near OHIS
12	Soil w/ vegetation	41°57'26"N, 21°29'32"E	Near OHIS
13	Soil w/ vegetation	41°57'40"N, 21°28'58"E	Near OHIS
14	Soil w/ vegetation	41°57'23.0"N 21°29'24.1"E	Near OHIS
15	Soil w/ suspect mycelia	41°57'44.0"N 21°29'24.8"E	Near OHIS
16	Pure soil	41°55'07"N, 21°29'49"E	Third landfill
17	Pure soil	41°55'07"N, 21°29'51"E	Third landfill
18	Pure soil	41°54'56"N, 21°31'43"E	Third landfill
19	Soil w/ suspect mycelia	41°55'06"N, 21°29'48"E	Third landfill

Legend: "Soil w/ vegetation" denotes the presence of root structures or detritus in the soil sample; "Soil w/ suspect mycelia" denotes that the sample had visible filamentous structures distinct from roots; "Pure soil" denotes a clean soil sample.

3. RESULTS AND DISCUSSIONS

A total of 39 unique strains of filamentous fungi were determined across the 19 samples. Out of the total 39 strains, 4 were shown to belong to the phylum Basidiomycota (Trivedi et al. 2010), notably isolates 6 and 12 belong to the order Polyporales and isolates 34 and 39 belong to the order Agaricales. The attained data was supplemented by constructed similes, grouping the isolates based on their mutual similarities. In this regard, isolates 5, 9, 33 and 35 showed several similarities to the genus *Fusarium* as defined by Leslie and Summerrell (2006) and by Nelson et al. (1983).

Isolates 4, 31 and 32 showed identical cultural and morphological phenotypes as the moulds belonging to the genus *Aspergillus* as defined by McClenny (2005) and by by Zafar et al. (2017).

Isolates 18, 19, 29 and 30 were most similar to moulds of the genus *Rhizopus* as evidenced by identical characteristics as those posed by Zafar et al. (2017).

However these construed similes serve very little deterministic value in lieu of preferable molecular and phylogenetic taxonomic methods, as fungi in particular are known to possess a discrepancy between genotype and phenotype identity, affording them their unmistakable adaptive capacity (Cappucino and Sherman 2002; Zafar et al. 2017). The key function of the garnered data in this study is, instead, focused on noting physiognomic categories and groupings of the filamentous fungi present in HCH contaminated soils in an attempt to better elucidate the adaptive trends of these tenacious inhabitants.

The raw data concerning the presence of these qualitative physiognomic categories, notably hirsuteness and septation, is shown in table 3. In comparison, figure 2 shows a visual representation of the emergent dominance of septate hirsute strains among the unique strains colonizing HCH contaminated soils.
The aforementioned morphological similes established to predefined fungal taxa such as *Fusarium* spp. (Sagar and Singh 2011) and *Rhizopus* (Russo et al. 2019b) spp. may hint at potential applicability of the isolated fungal strains in bioremediation assays, serving as a robust genotype-insensitive tool to select promising strains for further evaluation.

4. CONCLUSIONS

In summary, 19 soil samples were acquired from HCH contaminated soils from OHIS, Skopje. From these samples, 39 unique strains of filamentous fungi were isolated utilizing a series of continuous subcultivations along an incrementally increasing HCH concentration gradient. The isolated strains were compared amongst each other and in-group as well as between-group variance was observed. The qualitative morphological and cultural characteristics of each strain served to place each strain within predefined physiognomic categories. Further research on the metabolic capabilities of these fungi will afford a deeper statistical value to the co-adaptive nature of these physiognomic qualities and certain metabolic pathways. Notably, further study may reveal correlation between these groups and the HCH bioremediation potential of the fungi constituents of these groupings.

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