Original Research Paper

Characterization of Fungal Communities in Limed and Unlimed Lands Contaminated with Metals: Phospholipid Fatty Acid (PLFA) Analysis and Soil Respiration

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Abstract: Northern Ontario (Canada) especially the Greater Sudbury region is highly known for its nickel, copper and other metal deposits. The mining, roasting and smelting of these elements have caused disastrous effects on the vegetation and overall environment. Dolomitic lime which contains calcium and magnesium carbonate was applied to soils from 1980 to 1995 at different locations across Northern Ontario. The objective of the present study is to determine fungi diversity and abundance in selected limed and unlimed areas contaminated with metals in the Region. Soil respiration, fungi cultures and Phospholipid Fatty Acid (PLFA) were analyzed. The liming did maintain an increase in soil pH from extremely acid to slightly acid, even 25 to 30 years after liming applications. A total of 52 fungi species belonging to 34 genera were identified on growth media. The majority of fungi (up to 70%) in all the sites belong to the Ascomycota phylum. Some species were specific to one or two sites, while others were present in the majority of the sites. Fungal diversity and abundance were higher in limed soils compared to unlimed samples based on SDA medium growth. The rates of soil respiration in limed sites were also higher compared to unlimed areas. Phospholipid Fatty Acid (PLFA) analysis revealed a significantly higher total microbial biomass in samples from limed areas compared to unlimed samples. Total and arbuscular mycorrhizal fungi abundance based on this analysis followed the same trend. Surprisingly, there were 7 to 10 fold more bacteria than fungi in all the sites. Moreover, there was twice more Gram (-) bacteria than Gram (+) indicating that the sites are still severely stressed. Soil pH appears to be the most important factor for microbial abundance, diversity and activities than total metal content.

Keywords: Soil Liming, Fungi Diversity and Abundance, Phospholipid Fatty Acid, Soil Respiration, Northern Ontario

Introduction

Mining and smelting processes and activities have caused a variety of negative effects to the Northern Ontario environment. SO_2 fumigation and the accumulation of numerous metal particulates have damaged soil quality and ultimately, the vegetation and fungi populations inhabiting the region (Winterhalder, 1996). Balsillie *et al*. (1978) have documented a decrease in soil fungi and bacteria near the location of smelter sites within the Greater Sudbury Region. They also reported a decrease of the levels of colonization of roots of oxeye daisy (*Chrysanthemum leucanthemum*) and devil's paintbrush (*Hieracium aurantiacum*) by vesicular arbuscular mycorrhizal fungi in the Sudbury barrens (Balsillie *et al*., 1978).

A decrease in industrial emissions and remediation projects were initiated to restore Northern Ontario ecosystems (Winterhalder, 1996; Nkongolo *et al*., 2013; Theriault *et al*., 2013). Remediation consisted of neutralizing soil pH with the addition of lime as well as adding fertilizer, seed distribution and tree planting

(Winterhalder, 1996). In fact, dolomitic lime which contains calcium and magnesium carbonate was applied to soils from 1980 to 1995 at different locations across Northern Ontario. This resulted in an increase of organic matter that requires adequate microbial biomass for mineralization's processes. The intricacies of soil-fungal interactions are crucial in determining fungi diversity and abundance for a particular region. Thus, soil chemistry, metal binding capacity and metal speciation within the soil are key concepts that need to be adequately assessed and properly understood in order to sufficiently identify metal-fungal dynamics for a specific region. The examination of fungal diversity for a particular area is critical as it is an essential factor in the determination of their sustainability (Nielsen and Winding, 2002). Additionally, fungal abundance is another prime indicator of their population health (Nielsen and Winding, 2002). Since fungi play such a crucial role in soil formation, organic matter decomposition as well as in the transformations of toxic metabolites found in the soil (Ritz and Young, 2004), soil amendment strategies adopted in damaged ecosystems, such as liming, require extreme diligence to ensure that all fungal nutrient, metal, substrate requirements are addressed and achieved.

The objective of the present study is to determine fungi diversity and abundance in selected limed and unlimed areas contaminated with metals in the Northern Ontario Region. Soil respiration, fungi cultures and Phospholipid Fatty Acid (PLFA) were analyzed.

Materials and Methods

Soil Sampling and Processing

Soil sampling was completed as described in Goupil and Nkongolo (2014) at seven sites that include Wahnapitae Hydro-Dam, Daisy Lake, Kingsway, Kelly Lake, St.Charles (Hagar), Onaping Falls and Capreol (Fig. 1). Each sample was dried at room temperature (25°C) for 48 h. It was then sifted with a 2 mm sieve to remove all forms of debris. Three soil samples from each of the limed, unlimed or reference sites were mixed to produce a 'master' limed, unlimed or reference soil sample to be used later for fungal analysis. These "master mix" samples were used for serial dilutions. Only the top 5 cm (LFH layer) of organic soil was analyzed because it was hypothesized that the majority of fungi colonies would be present within this soil horizon.

Fungal Isolation Method

All materials, except the soil samples, were autoclaved before use. Serial dilutions were subsequently prepared. For that purpose, 10.0 g of soil was mixed with 90.0 mL of sterile H_2O resulting in a dilution of 1/10. A sterile stopper was then firmly placed on the Erlenmeyer flask containing the 1/10 dilution. The solution was shaken vigorously for 1 h. Four successive dilutions by a factor of 10 were then prepared. They include 1/100, 1/1000, 1/10000, 1/100000 and 1/1000000.
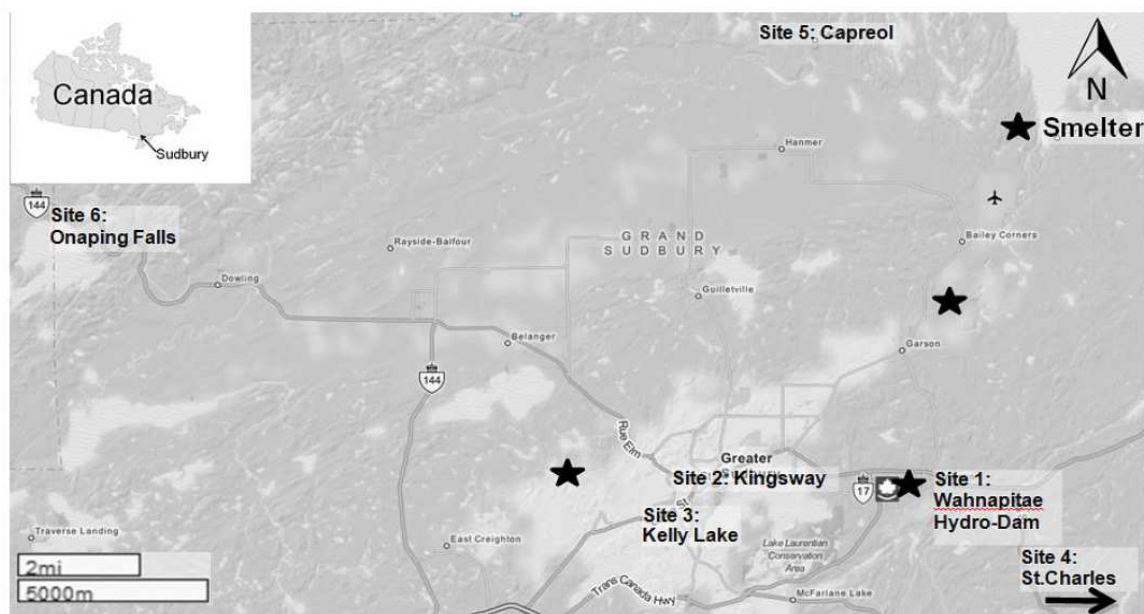

Fig. 1. Locations of sampling areas from the Greater Sudbury Region. Site 1: Wahnapitae Hydro-Dam; Site 2: Kingsway; Site 3: Kelly Lake; Site 4: St.Charles (Hagar); Site 5: Capreol and Site 6: Onaping Falls. Sites 4, 5 and 6 were used as reference sites. St.Charles (Hagar) is outside the map as a reference site and is about 50-60 km from Sudbury. Source: Edited from Google Map 2014

Two medium types were used for this study, namely Sabouraud Dextrose Agar (SDA) and Malt Extract Agar (MEA). SDA is a multipurpose growing medium for numerous fungal species whereas, MEA is an ideal medium for the cultivation of yeasts and molds. For each medium (SDA and MEA), six plates including a control and different diluted solutions were prepared and duplicated. The labelled plates were inoculated with 0.1 mL of each solution respectively. The inocula were evenly spread over the surface of the agar plate using a sterile glass rod. The plates were then incubated at 26°C until colony masses were easily observed. The plates were examined every day starting at 3-4 days after incubation.

Slide Preparation

All standard microscope slides were cleaned with 95% ethanol. A few drops of lactophenol cotton blue were then placed on the slides. With the aid of a pin probe, parts of a fungal colony were placed onto the slide. A cover slip was then placed onto the mount. Slides were properly labeled and air dried before storage. Fungi identification was performed using classification guides and was based on microscopic morphology and colony appearance on the media. The reference guides included detection and isolation of soil fungi (Davet and Rouxel, 2000); Compendium of soil fungi (Domsch *et al.*, 2007); Identification of fungi: An illustrated introduction with keys, glossary and guide to literature (Dugan, 2006); A laboratory guide to common *Penicillium* species (Pitt and Hocking, 1985); Pictorial atlas of soil and seed fungi: Morphologies of cultured fungi and key to species (Watanabe, 2002); and the Mucorales (Zycha and Siepmann, 1970).

Phospholipid Fatty Acid (PLFA) Analysis and Soil Respiration

Phospholipid Analysis (PLFA) was performed at FAME Lab, Microbial ID. Inc, Newark, Delaware (USA) as described in Buyer and Saaser (2012). Soil respiration was assessed using the Solvita Soil Test as described in Goupil and Nkongolo (2014). Soil samples were dried in an incubator at 45°C for 24 h prior to the test. Dried soil samples were weighed into a capillary cup after placing a fiber filter in the bottom of the cup. Each beaker, containing 40 g of dried soil, was placed into a glass jar with the use of forceps. Following this, 20 mL of distilled water was placed into the glass jar. A CO_2 probe was then inserted into the glass jar using forceps. Jars were opened 24 h later to remove the probes. Probe color was determined using a Digital Color Reader (DCR) as per the manufacturer instructions. Interpretations of the DCR provided data were based on Solvita's overall guidelines.

Statistical Analysis

Fungal diversity and abundance in unlimed and limed soil samples were compared using Student t-tests. One-Way Analysis of Variance (ANOVA) tests were also conducted between limed, unlimed and reference soil samples for fungal diversity and abundance. Individual differences were determined using Tukey's multiple range test.

Results

Soil pH Analysis

The pH in unlimed sites was consistent with that documented for soils on coarser textured soils with coniferous vegetation on the Canadian Shield of at <4, classified as extreme acid (15, 16). The pH in limed sites was significantly higher ranging from 4.12 to 6.75 in the top organic layer compared to unlimed sites (Table 1). The liming did maintain an increase in soil pH from extremely acid to slightly acid, even 20 to 35 years after liming applications.

Fungi Diversity and Abundance

Figure 2 shows the long term effect of liming on plant vegetation and population complexity. All fungal species identified in limed and unlimed sites as well as in the reference sites are described in Fig. 3-5. Venn diagrams (Fig. 3-5) also show different fungi species and their shared or specific site location. Mean number of fungi and mean level of fungi abundance for limed, unlimed and reference sites are presented in Table 2 and 3. The most abundant fungi species are described in Table 4.

Table 1. Mean pH for limed, unlimed and reference sites in Northern Ontario

Site	pH H_2O	pH $CaCl_2$
Kelly Lake limed	6.41	6.13
Kelly Lake unlimed	3.75	3.37
Kingsway limed	4.67	4.35
Kingsway unlimed	3.87	2.35
Wahnapitae Hydro-Dam limed	6.75	6.34
Wahnapitae Hydro-Dam unlimed	3.82	3.56
Capreol (reference site)	3.92	3.43
St.Charles (Hagar) (reference site)	3.50	3.23
Onaping Falls (reference site)	4.84	3.92

Table 2. Number of different fungi species within medium for limed, unlimed and reference sites

	Number of species (Mean ± SE)*	
Treatment	SDA	MEA
Limed sites	7.25 a ±0.53	5.13 a ±0.95
Unlimed sites	5.13 b ±0.44	5.63 a ±0.42
Reference sites	5.83 ab ±0.31	7.50 a ±0.85

Limed and unlimed sites include: Wahnapitae Hydro-Dam, Daisy Lake, Kingsway and Kelly Lake. Reference sites include: St.Charles (Hagar), Onaping Falls and Capreol
*Means in columns with a common letter are not significantly different based on Tukey multiple comparison test (p≥0.05). SE = Standard error

Fig. 2. Wahnapitae Hydro-Dam site (a) Limed area and (b) Unlimed area

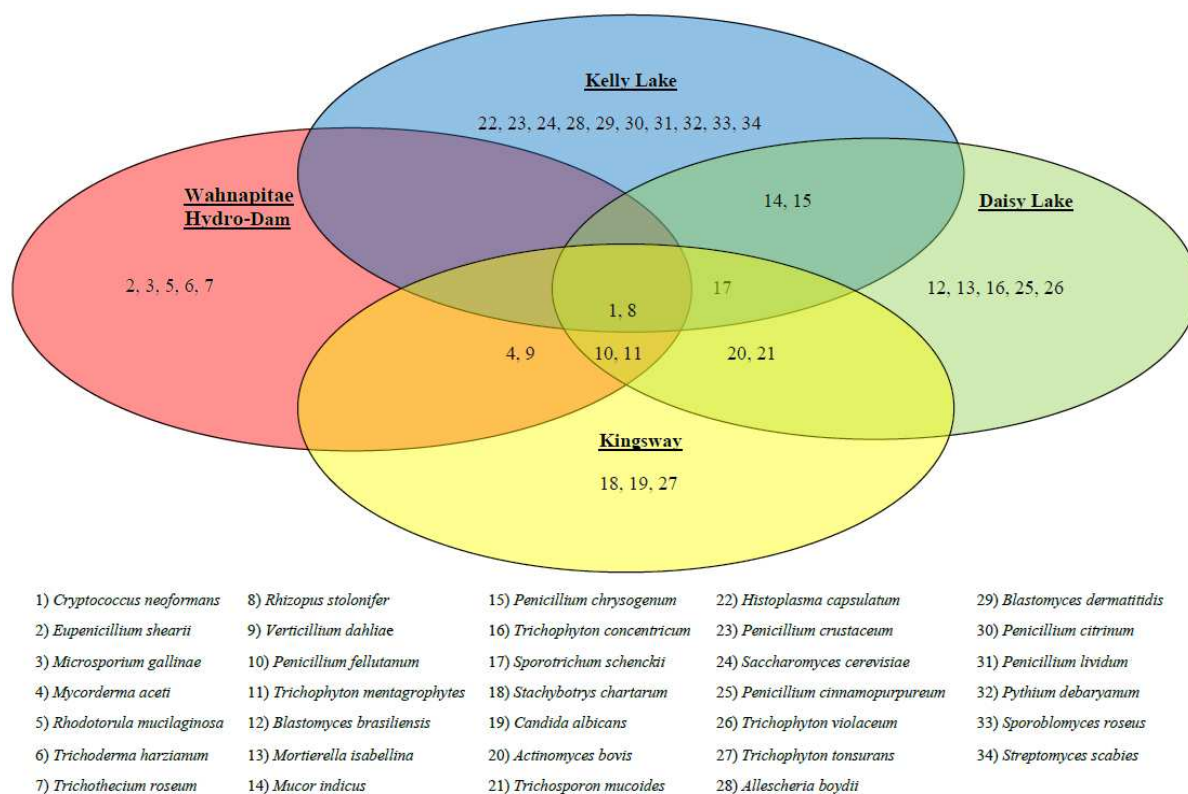| 1) *Cryptococcus neoformans* | 8) *Rhizopus stolonifer* | 15) *Penicillium chrysogenum* | 22) *Histoplasma capsulatum* | 29) *Blastomyces dermatitidis* |
2) *Eupenicillium shearii*	9) *Verticillium dahliae*	16) *Trichophyton concentricum*	23) *Penicillium crustaceum*	30) *Penicillium citrinum*
3) *Microsporium gallinae*	10) *Penicillium fellutanum*	17) *Sporotrichum schenckii*	24) *Saccharomyces cerevisiae*	31) *Penicillium lividum*
4) *Mycorderma aceti*	11) *Trichophyton mentagrophytes*	18) *Stachybotrys chartarum*	25) *Penicillium cinnamopurpureum*	32) *Pythium debaryanum*
5) *Rhodotorula mucilaginosa*	12) *Blastomyces brasiliensis*	19) *Candida albicans*	26) *Trichophyton violaceum*	33) *Sporoblomyces roseus*
6) *Trichoderma harzianum*	13) *Mortierella isabellina*	20) *Actinomyces bovis*	27) *Trichophyton tonsurans*	34) *Streptomyces scabies*
7) *Trichothecium roseum*	14) *Mucor indicus*	21) *Trichosporon mucoides*	28) *Allescheria boydii*	

Fig. 3. Venn diagram illustrating common fungi species among limed areas based on SDA and MEA medium analysis

A total of 52 fungi species belonging to 34 genera were identified. Some species were specific to one or two sites, while others were present in the majority of the sites including the reference sites. A total of 23 fungi species were identified in samples from limed areas based on fungi growth on SDA medium plates, while 17 species were observed in samples from unlimed sites. A total of 18 fungi species were found in the reference sites. These results indicate that fungal diversity is higher in limed soils compared to unlimed soils based on SDA medium growth (Table 2).
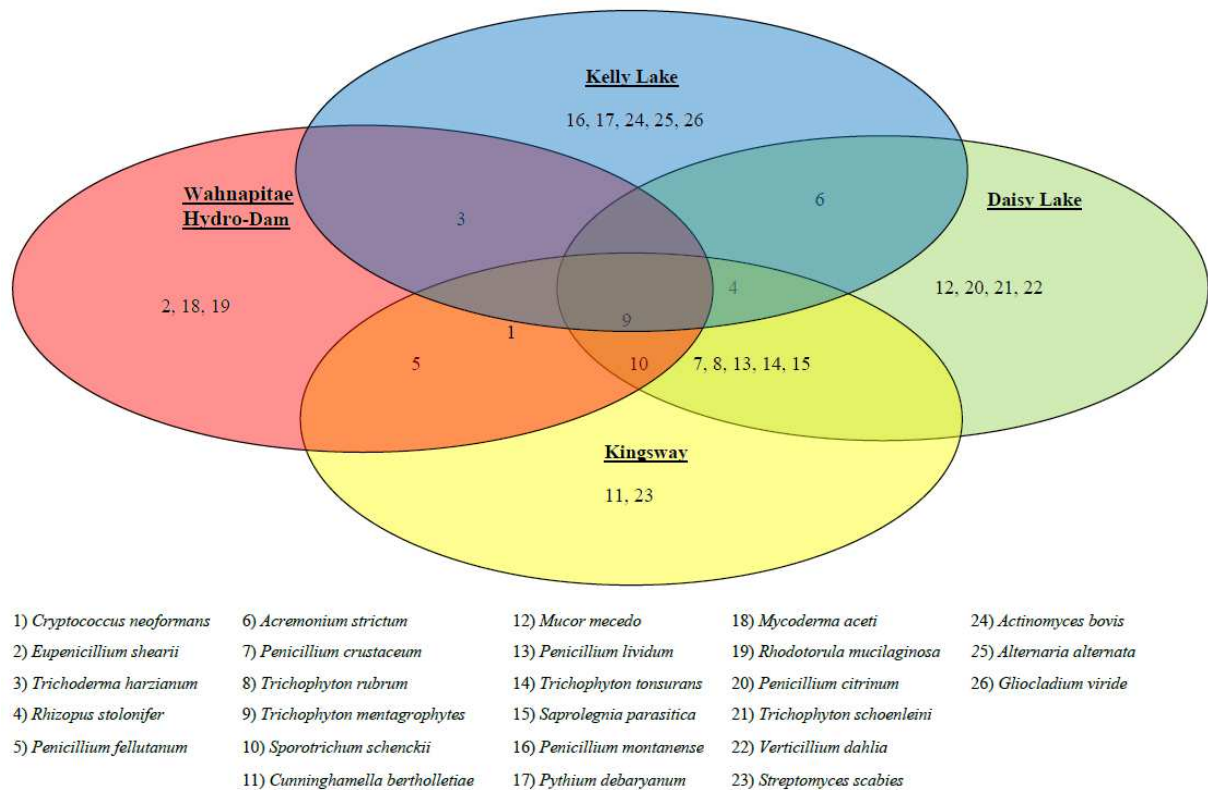
1) *Cryptococcus neoformans*
2) *Eupenicillium shearii*
3) *Trichoderma harzianum*
4) *Rhizopus stolonifer*
5) *Penicillium fellutanum*
6) *Acremonium strictum*
7) *Penicillium crustaceum*
8) *Trichophyton rubrum*
9) *Trichophyton mentagrophytes*
10) *Sporotrichum schenckii*
11) *Cunninghamella bertholletiae*
12) *Mucor mecedo*
13) *Penicillium lividum*
14) *Trichophyton tonsurans*
15) *Saprolegnia parasitica*
16) *Penicillium montanense*
17) *Pythium debaryanum*
18) *Mycoderma aceti*
19) *Rhodotorula mucilaginosa*
20) *Penicillium citrinum*
21) *Trichophyton schoenleini*
22) *Verticillium dahlia*
23) *Streptomyces scabies*
24) *Actinomyces bovis*
25) *Alternaria alternata*
26) *Gliocladium viride*

Fig. 4. Venn diagram illustrating common fungi species among unlimed areas based on SDA and MEA medium analysis

1) *Cunninghamella bertholletiae*
2) *Eupenicillium shearii*
3) *Mucor indicus*
4) *Penicillium crustaceum*
5) *Trichophyton mentagrophytes*
6) *Allescheria boydii*
7) *Candida glabrata*
8) *Candida lusitaniae*
9) *Cryptococcus neoformans*
10) *Penicillium citrinum*
11) *Penicillium decumbens*
12) *Absidia corymbifera*
13) *Blastomyces brasiliensis*
14) *Blastomyces dermititidis*
15) *Cladosporium phaerospermum*
16) *Penicillium montanense*
17) *Trichoderma harzianum*
18) *Trichophyton tonsurans*
19) *Rhizopus stolonifer*
20) *Streptomyces scabies*
21) *Trichophyton concentricum*
22) *Coccidioides immitis*
23) *Penicillium citreonigrum*
24) *Pythium debaryanum*
25) *Rhodotorula mucilaginosa*
26) *Trichophyton faviforme*
27) *Trichosporon mucoides*
28) *Epicoccum purpurascens*
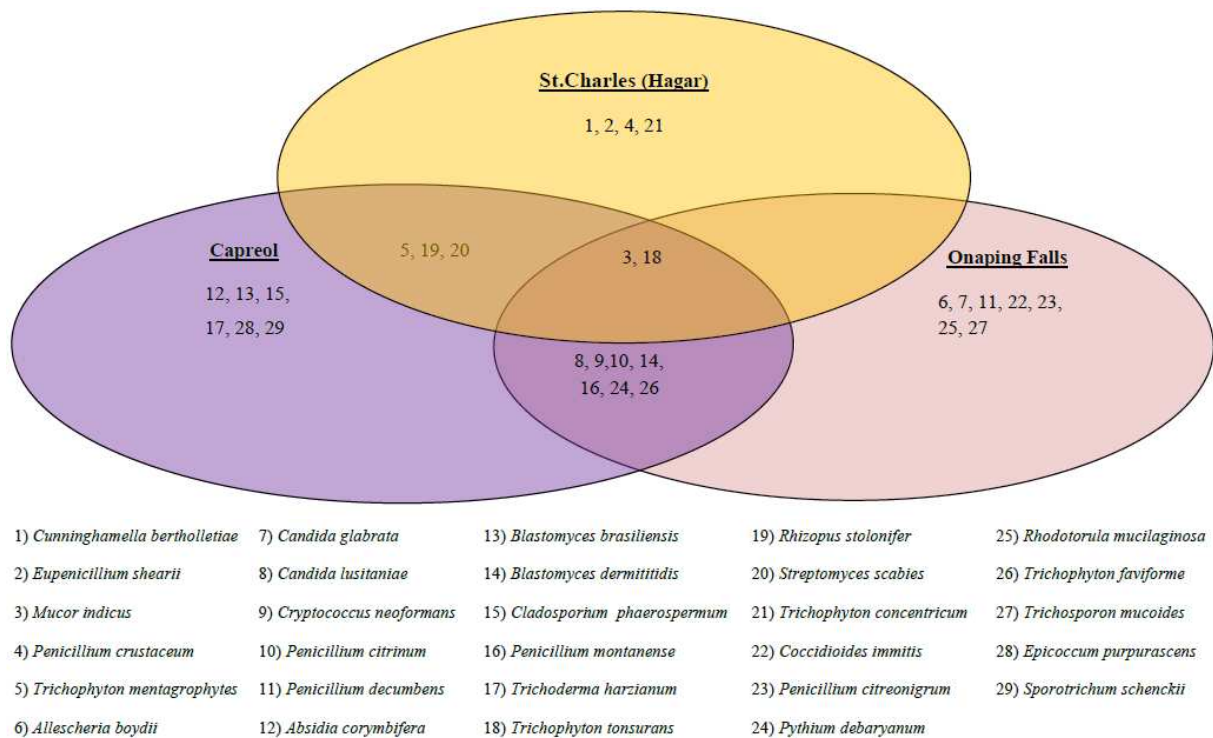29) *Sporotrichum schenckii*

Fig. 5. Venn diagram illustrating common fungi species among reference sites based on SDA and MEA medium analysis

No significant difference was observed for fungi diversity based on MEA medium growth. Overall, fungi growth on the MEA medium revealed the presence of 21 species in samples from limed areas and 19 species from unlimed areas. A total of 20 species were found in the three reference sites based on MEA growth analysis.

Fungi Abundance Based on Medium

Optimal fungal growth was observed on SDA medium at dilution factors of 10-1 to 10-4 and at 10-1 to 10-3 dilution factors for the MEA medium. In both media, the majority of the fungal growth was observed within the first 7 to 10 days post inoculation. Table 4 described for each site the species that was the most abundant based on SDA and MEA growth. The most prevalent fungi species on the SDA medium include *Cryptococcus neoformans, Acremonium strictum, Candida albicans, Cunninghamella bertholletiae, Penicillium fellutanum, Sporotrichum schenckii, Trichophyton mentagrophytes* and *Penicillium citrinum*. For the MEA medium, *Cryptococcus neoformans, Streptomyces scabies, Sprorotrichum schenckii, Acremonium strictum, Mycoderma aceti, Penicillium montanense, Trchosporon mucoides, Sporotrichum schenckii* and *Trichophyton tonsurans* were the most prevalent species. In general, 41 species were identified on SDA medium and 36 species on MEA medium.

Fungi Species Distribution in Limed and Unlimed Areas

Distribution Among Sites

Fungi species distribution was based on growth on SDA and MEA media. For limed sites, 70% of the fungi species belong to the Ascomycota phylum, 12% Basidiomycota, 9% Zygomycota, 6% Deuteromycota and 3% Oomycota. At the species level, *Cryptococcus neoformans* and *Rhizopus stolonifer* were found in all the areas. Five fungi species were specific to Wahnapitae Hydro-Dam, three different species specific to Kingsway, five to Daisy Lake and ten to Kelly Lake sites (Fig. 3).

For unlimed sites, 65% of the fungi species belong to the Ascomycota phylum, 8% Basidiomycota, 11% Zygomycota, 8% Deuteromycota and 8% Oomycota. *Trichophyton mentagrophytes* was a common species to all the areas, while three species were specific to Wahnapitae Hydro-Dam, two to Kingsway, four to Daisy Lake and five to Kelly Lake sites. Other species were present in some sites and absent in others (Fig. 4).

Table 3. Mean fungi abundance in soil samples from limed, unlimed and reference sites based on growth on SDA and MEA media

Treatment	Abundance (Mean c.f.u./g. ± SE)*	
	SDA	MEA
Limed sites	1.82×10^8 a	1.54×10^8 a
	$\pm 5.17 \times 10^7$	$\pm 7.84 \times 10^7$
Unlimed sites	8.11×10^7 b	1.61×10^8 a
Reference sites	$\pm 1.73 \times 10^7$	$\pm 4.44 \times 10^7$
	4.0×10^8 a	1.36×10^9 b
	$\pm 1.22 \times 10^8$	$\pm 8.43 \times 10^8$

Limed and unlimed sites include: Wahnapitae Hydro-Dam, Daisy Lake, Kingsway and Kelly Lake. Reference sites include: St.Charles (Hagar), Onaping Falls and Capreol
*Means in columns with a common letter are not significantly different based on Tukey multiple comparison test ($p \geq 0.05$). SE = Standard Error

Table 4. Fungi abundance

Site	Most abundant species	Abundance (C.F.U./g)
SDA medium		
Daisy Lake Limed	*Sporotrichum schenckii*	3.38×10^6
Daisy Lake Unlimed	*Acremonium strictum*	5.26×10^7
Kingsway Limed	*Candida albicans*	1.12×10^8
Kingsway Unlimed	*Cunninghamella bertholletiae*	3.42×10^7
Kelly Lake Limed	*Cryptococcus neoformans*	2.66×10^8
Kelly Lake Unlimed	*Cryptococcus neoformans*	1.06×10^8
Wahnapitae Hydro-Dam Limed	*Penicillium fellutanum*	7.77×10^7
Wahnapitae Hydro-Dam Unlimed	*Trichophyton mentagrophytes*	1.22×10^7
Reference sites		
St.Charles (Hagar)	*Trichophyton mentagrophytes*	1.01×10^8
Onaping Falls	*Penicillium citrinum*	2.79×10^8
Capreol	*Trichophyton mentagrophytes*	1.05×10^8
MEA medium		
Daisy Lake Limed	*Sporotrichum schenckii*	6.18×10^6
Daisy Lake Unlimed	*Acremonium strictum*	1.37×10^8
Kingsway Limed	*Mycoderma aceti*	6.60×10^7
Kingsway Unlimed	*Cryptococcus neoformans*	1.66×10^8
Kelly Lake Limed	*Streptomyces scabies*	4.89×10^8
Kelly Lake Unlimed	*Penicillium montanense*	3.86×10^8
Reference sites		
St.Charles (Hagar)	*Trichophyton tonsurans*	1.59×10^8
Onaping Falls	*Trichosporon mucoides*	1.23×10^8
Capreol	*Sporotrichum schenckii*	1.61×10^8

For reference sites, 69% of the fungi species belong to the Ascomycota phylum, 10% Basidiomycota, 14% Zygomycota, 3% Deuteromycota and 4% Oomycota. At the species level, *Mucor indicus* and *Trichophyton tonsurans* were common to all the sites. Six species where specific to Capreol reference site, seven to Onaping Falls and four to St.Charles (Hagar) (Fig. 5).

Distribution within Sites

SDA Medium

For Wahnapitae Hydro-Dam site, *Penicillium fellutanum, Rhodotorula mucilaginosa, Trichoderma harzianum* and *Trichophyton mentagrophytes* were the most commonly found in limed areas while *Eupenicillium shearii, Penicillium fellutanum, Sporotrichum schenckii* and *Trichophyton mentgrophytes* were the most frequent in unlimed sites.

For Daisy Lake, *Mortierella isabellina, Penicillium chrysogenum* and *Sporotrichum schenckii* were most common species in limed site while *Acremonium strictum, Penicillium lividum* and *Rhizopus stolonifer* were the most prevalent in unlimed sites.

For Kingsway, *Candida albicans, Penicillium fellutanum, Rhizopus stolonifer* and *Trichophyton mentagrophytes* were the most frequent while *Cunninghamella bertholletiae* and *Saprolegnia parasitica* were the most common in unlimed sites.

For Kelly Lake, *Cryptococcus neoformans, Penicillium crustaceum* and *Sporotrichum schenckii* were the most abundant while *Cryptococcus neoformans* and *Rhizopus stolonifer* were the most frequent in unlimed sites.

For the reference site, *Cunninghamella bertholletiae, Eupenicillium shearii, Mucor indicus, Penicillium crustaceum* and *Trichophyton mentagrophytes* were the most common in St.Charles (Hagar); *Allescheria boydii, Candida glabrata, Candida lusitaniae, Cryptococcus neoformans, Mucor indicus, Penicillium citrinum* and *Penicillium decumbens* in Onaping Falls; *Absidia corymbifera, Blastomyces brasiliensis, Blastomyces dermatitidis, Cladosporium sphaerospermum, Mucor indicus, Penicillium montanense, Trichoderma harzianum, Trichophyton mentagrophytes* and *Trichophyton tonsurans* in Capreol.

MEA Medium

For Wahanapitae Hydro-Dam, *Mycoderma aceti* and *Trichophyton mentagrophytes* were more common in limed area while *Cryptococcus neoformans, Mycoderma aceti* and *Trichophyton mentagrophytes* were most frequent in unlimed site.

For Daisy Lake, *Penicillium chrysogenum, Sporotrichum schenckii* and *Trichophyton mentagrophytes* were the most abundant in limed area while *Acremonium strictum, Saprolegnia parasitica*

and *Verticillium dahlia* were the most frequent in unlimed site.

For Kingsway, *Mycoderma aceti* and *Trichophyton mentagrophytes* were the most common while *Cryptococcus neoformans, Saprolegnia parasitica, Sporotrichum schenckii* and *Trichophyton mentagrophytes* were most frequent in unlimed site.

For Kelly Lake, *Penicillium lividum* and *Streptomyces scabies* were the most abundant in limed area while *Acremonium strictum, Actinomyces bovis* and *Alternaria alternata* were the most prevalent in unlimed site.

For reference sites, *Penicillium crustaceum, Rhizopus stolonifer, Streptomyces scabies, Trichophyton concentricum, Trichophyton mentagrophytes* and *Trichophyton tonsurans* were the most common in St.Charles (Hagar); *Blastomyces dematitidis, Coccidioides immitis, Mucor indicus, Penicillium citreonigrum, Penicillium citrinum, Penicillium montanense, Pythium debaryanum, Rhodotorula mucilaginosa, Trichophyton faviforme, Trichophyton tonsurans* and *Trichosporon mucoides* in Onaping Falls; and *Blastomyces dermatitidis, Candida lusitaniae, Cryptococcus neoformans, Epicoccum purpurascens, Penicillium citrinum, Pythium debaryanum, Rhizopus stolonifer, Sporotrichum schenckii, Streptomyces scabies* and *Trichophyton faviforme* in Capreol.

PLFA Analysis

Results of phospholipid Fatty acid analysis are described in Table 5. This analysis revealed higher microbial biomass in samples from limed areas compared to unlimed samples. Significant differences were also observed between limed and unlimed site for total fungi and arbuscular mycorrhizal fungi abundance based on the PLFA analysis. In general there more bacteria than fungi in all the sites analyzed (Table 5). In fact total bacteria represent 68.4, 74.3 and 71.4% for unlimed, limed and references sites, respectively. These values were only 9.8% (for unlimed), 6.7% (for limed) and 9.4% (for reference sites) for total fungi biomass. The ratio between fungi and bacteria was very low for all the groups. A sensitive difference between fungi/bacteria ratio for limed and unlimed sites was observed (Table 6). There were twice more Gram (-) bacteria than Gram (+) in all the sites (Table 5).

Soil Respiration

There was significant higher soil respiration rates in limed sites compared to unlimed areas. Respiration rates for the reference sites were similar to those documented for the limed sites (Table 5).

Table 5. Total microbial biomass, fungi and AM fungi biomass and soil respiration in limed and unlimed sites in Northern Ontario using phospholipid fatty acid analysis and Solvita test*

Site	Total microbial biomass	AM Fungi (ng/g)	Fungi (ng/g)	Fungi /bacteria ratio	Gram (-) bacteria (ng/g)	Gram (+) bacteria (ng/g)	CO_2-C (mg/kg (ppm)) Summer	Fall
Unlimed sites	145.72a±33.73	6.1a±1.00	11.8a±7.0	0.18	67.1a±6.49	37.3a±3.42	57.0a	52.3a
Limed sites	339.63b±83.25	14.9b±1.8	26.7b±4.07	0.14	174.6b±1.9	76.1b±3.94	69.3b	71.0b
Reference sites	431.81b±80.36	19.4b±1.6	42.5b±4.37	0.16	212.8b±5.66	95.3b±0.52	74.5b	69.0b

Unlimed and limed sites: Wahnapitae Hydro-Dam, Kingsway and Kelly Lake; Reference sites: Onaping Falls, Capreol and St.Charles (Hagar).
*Means in columns with a common letter are not significantly different based on Tukey multiple comparison test (p≥0.05)

Table 6. Phospholipid Fatty Acid (PLFA) ratios analyzed in soil samples from the Greater Sudbury Region

Sites	Fungi/ Bacteria	Predator/ Prey	Gram positive/ Gram negative	Saturated/ Unsaturated	Mono/ Poly	16w/16 cyclo	18w/19 cyclo
Unlimed sites	0.18	0.04	0.74	1.17	3.82	2.43	0.80
Limed sites	0.14	0.02	0.55	0.77	6.44	2.64	2.30
Reference sites	0.16	0.05	0.48	0.74	4.00	3.00	1.12

Discussion

Soil Metal Content

Detail of soil metal analysis has been described in (Nkongolo *et al.*, 2013; Goupil and Nkongolo, 2014; Tran *et al.*, 2014). Hence no metal analysis was conducted in the present study. These previous reports show no significant differences between limed and unlimed areas with regards to total element concentrations with the exception of arsenic. As expected, the limed samples contained higher levels of calcium and magnesium compared to unlimed sites. Based on these studies, there were significantly higher contents of bioavailable aluminum, iron, manganese and strontium in unlimed sites compared to limed area. The levels of bioavailable cobalt, copper, total metals that was bioavailable was very small (Nkongolo *et al.*, 2013; Goupil and Nkongolo, 2014; Tran *et al.*, 2014).

Soil Characteristics, Fungi Abundance and Diversity

Fungi diversity and abundance in a region are generally tribute to soil chemical and physical conditions. Smith and Doran (1996) reported that fungi prefer acidic soil since their growth was generally observed in the soil with a pH between 2 and 7 with an optimum of 5. However, some fungi have a better growth rate in alkaline soils compared to acidic soils (Kendrick, 2000). In fact, soil pH affects many biological processes. For example, organic matter mineralization is slowed down or stopped at highly acidic or alkaline pH levels, which is attributed to poor microbial activity linked to bacteria (Smith and Doran, 1996). At low pH levels, nitrification and nitrogen fixation will also be inhibited (Smith and Doran, 1996). Furthermore, the solubility of heavy metals as well as the mobility and degradation of herbicides and insecticides are pH dependant (Smith and Doran, 1996). Soil pH affects cation availability which in turn influences aggregate stability because multivalent cations, like calcium ions, act as bridges between organic colloids and clays (Smith and Doran, 1996).

The effects of soil liming include an intricate change in the total nutrient availability in the soil as well as an increase in soil pH (Mengel and Kirkby, 1987). Some nutrients prove more useful for some species of fungi than others and the levels of such macro and micro nutrients in the substrate dictate the survival of fungi species (Kendrick, 2000). Fungi use a variety of metals to accomplish their vital processes. In the present study, the total and bioavailable concentration of nutrients and metal elements in the limed and unlimed organic surface horizons (LFH) of soils were determined to better understand their role on fungi sustainability. Elemental analysis revealed significantly lower concentrations of total arsenic and bioavailable aluminium, iron and strontium in limed sites compared to unlimed sites. As expected, high levels of bioavailable calcium and magnesium were found in limed sites reflecting the addition of the dolomitic and calcitic limestone that was applied 30-40 years ago.

Fungi have the ability to use many metals or elements as food sources while other organisms prove to be intolerant to many elements in their soil environment even at low concentrations. It was difficult to estimate if the low level of bioavailable metals could have any effect in fungi diversity and abundance.

While sharing the common characteristics of Canadian Shield soils, some sites are better suited for some phyla of fungi to grow and thrive based on their respective soil properties. This is why some fungi species are unique to some sites while others are found in all four targeted limed or unlimed sites. For example, for the limed soil, *Cryptococcus neoformans* and *Rhizopus stolonifer* were common to all the four targeted sites, namely Wahnapitae Hydro-Dam, Daisy Lake, Kingsway and Kelly Lake, while many other fungi species were site(s)-specific.

Climatic factors also influence fungi diversity as well as abundance within a region in general. Physical parameters such as temperature, light and even gravity have been found to have significant effects on different fungi species (Kendrick, 2000). Some psychrophilic fungi have been documented to grow at temperatures below 0°C while, some thermophilic fungi have the ability to grow and thrive at temperatures above 50°C (Kendrick, 2000). Some fungi species require light for fruiting while others are indifferent to illumination (Kendrick, 2000). Several macrofungi are extremely sensitive to gravity while numerous microfungi are totally oblivious to gravity (Kendrick, 2000). Consequently, because the sites of Wahnapitae Hydro-Dam, Daisy Lake, Kingsway, Kelly Lake, St.Charles (Hagar), Onaping Falls and Capreol are geographically separated and distant, they each have different topographic settings resulting in different temperatures and illumination rates (at times) which could have some effects on their respective fungi populations.

Fungi Growth in Culture Media

In nature, fungi can develop mechanisms to feed themselves from different substrates but when grown on media, nutrients must be added to ensure that fungal colonies will grow (Kendrick, 2000). Two fungal media, namely SDA and MEA, were utilized in this study. Both media are chemically different from one another and allow for variations in terms of fungal diversity and abundance. SDA, a multipurpose medium, is among one of the most widely used fungal media as it allows for numerous fungi species to grow. SDA medium contains sources of carbon, nitrogen as well as vitamins just like all fungal media utilized for commercial purposes. Among its' ingredients, SDA medium contains glucose (to be utilized as a carbon source) and neopeptone and polypeptone agar (utilized as a solidifying agent for nitrogen). SDA pH is around 5.6 which is slightly acidic and appropriate for the growth of a multitude of fungi species that have an optimal growth at a pH of 5 (Smith and Doran, 1996). Commercially available SDA can include antibiotics such as streptomycin, which does not harm fungi. Such formulations are known as "restrictive" or "selective" while SDA formulations that have no inhibitor are known as "permissive".

To document other possible fungi species growing in sites within Northern Ontario Region, MEA medium was also used. This medium is suited for the isolation, cultivation and enumeration of yeasts and molds. MEA medium provides sources of carbon, protein and nutrients that are necessary for the growth of microorganisms. Because MEA medium contains high concentrations of maltose and other saccharides as energy sources, it primarily allows for yeasts and molds to grow and to some extent some other fungi species.

But bacterial growth is restricted on petri dishes. MEA medium uses dextrin and glycerin as carbon sources and Peptone is utilized as a nitrogen source. Bacteriological agar is the solidifying agent for this particular strand of medium. MEA medium has a pH of 4.7.

Fungi Diversity and Abundance

A significantly high level of fungal diversity was found in limed soil samples compared to unlimed soil samples based on data from SDA growth essays. These data are consistent with (Smith and Doran, 1996) findings. An opposite trend was observed for the MEA medium where more fungal diversity was recorded in the unlimed soil samples compared to the limed ones. These results can be ascribed to chemical and pH differences between SDA and MEA media.

Variability in fungal abundance follows the same trend as the species diversity. A significantly higher level of fungal abundance was observed in the limed areas compared to the unlimed sites based on SDA medium data. These results support Weyman-Kaczmarkowa and Pedziwilk's (2000) findings documenting the effects of liming ($Ca(OH)_2$) on a number of fungal Colony-Forming Units (CFU). Although a different type of limestone was utilized in their study, they nevertheless recorded elevated mean fungal CFU numbers when soil pH reached 4.5 (Weyman-Kaczmarkowa and Pedziwilk, 2000) compared to more acidic soils.

For the MEA medium, an opposite trend was observed where more fungal abundance was recorded in the unlimed sites compared to the limed sites. This again can possibly be explained by the chemical ingredients and lower pH of the MEA medium which is better suited for yeast growth. Higher yeast counts were in fact documented for all sites in the MEA medium.

Soil Respiration and PLFA Analysis

Soil respiration is known as an ecosystem process by which carbon dioxide is released from the soil by means of root respiration, microbial decomposition of litter and soil organic matter as well as fauna respiration (Yiqi and Zhou, 2006). Soil respiration is intricately linked to the general processes occurring in ecosystems becasuse it is strongly related to ecosystem productivity, soil fertility, as well as regional and global carbon cycles (Yiqi and Zhou, 2006).

While the evaluation of soil respiration to determine soil health is widely adopted, the determination of the individual contributions of the different microorganisms to the total metabolism involved in the soil is rather difficult to undertake in terms of methodology (Pietikåinen *et al.*, 2005). However, according to a model developed by Anderson and Domsch (1974), bacterial and fungal respiration can indeed be calculated using

inhibitors. In the soil used to develop the method, they obtained a ratio of bacterial to fungal respiration of about 20/80 which supports the hypothesis that the fungal biomass dominates the metabolism of many agricultural and forest soils as previously mentioned (Anderson and Domsch, 1974). Biomass measurements by (Satchell, 1971; Shields *et al.*, 1973; Parkinson, 1973) and as cited by Clark and Paul (1970) also support Anderson and Domsch's (1974) findings. They indicated that soil biological biomass was dominated by the fungal component. Consequently, one could state that fungi are the major decomposers in these soils and as such, their diversity and abundance are crucial to their metabolic activity and ultimately, to soil respiration outputs for a specific region.

It should be pointed out that culture media analysis revealed the diversity and the abundance of fungi species that can grow on the two media used while PLFA tests estimate the amount of living fungi and other microorganisms. Many fungal species elude culturing in the laboratory (van Elsas *et al.*, 2000). To overcome problems associated with non-culturable fungi or bacteria, various methods have been developed to identify and study these organisms including fatty acid analysis and numerous DNA and RNA based methods (Kirk *et al.*, 2004). Fatty acids are the key component of cellular membrane of all living cells. Thus, PLFA provides an accurate census of the current living community. It is a culture-independent tool for investigating microbial populations in ecological studies (Hackl *et al.*, 2005; Kaur *et al.*, 2005).

But the results of the PFLA analysis in the present study show that bacteria are the main components of all the sites surveyed. no significant differences between limed and unlimed sites in total and arbuscular mycorrhizal fungi biomass. Significant differences between the two groups of sites (limed Vs unlimed) were rather observed for total microbial biomass, total fungi and arbuscular mycorrhizal. This clearly indicated that liming has significant long term effect on live microbial communities. The fungi/bacteria ratios were very low indicating a community of fungi/bacteria close to 1 indicates a balance and a system that is adapted to environmental conditions. Since the main lasting effect of liming is soil acidity, the results suggest that the increase in soil pH generated by liming could be more beneficial to bacteria than to fungi. Overall, there were twice as much Gram (-) bacteria than Gram (+) confirming that all the sites including the reference areas are still severely stressed. This finding does somewhat corroborate with Anderson and Domsch (1974) results. They reported that soil with a low pH such as in the present study showed the highest level of bacterial activity compared to neutral (pH = 7.5) soil.

Soil Microbial Abundance, Respiration and Metal Content

It has been demonstrated that fungi are more tolerant to heavy metals as a group in comparison to bacteria (Doelman, 1985; Hiroki, 1992). Furthermore, different biomass measurements or plate counting techniques have revealed that heavy metals will affect bacteria and fungi in a different manner in the soil (Hiroki, 1992; Khan and Scullion, 2002; Maliszewska *et al.*, 1985; Muller *et al.*, 2001). Consequently, metal speciation should affect soil respiration to some extent. Rajapaksha *et al.* (2004) reported that soil respiration rate was slightly affected by metal contamination and they found that a clear dose-response effect was encountered at added metal concentrations above 4 mmol kg^{-1}. In fact, they discovered that the highest levels of Copper and Zinc contamination resulted in a 30% reduction of soil respiration rate compared to the control soil (Rajapaksha *et al.*, 2004). Furthermore, the metal contamination of Zinc and Copper was not differentiated in terms of metal effect on soil respiration (Rajapaksha *et al.*, 2004). Previous reports also indicate that soil respiration rates in the metal-contaminated soils gradually decrease in comparison to the non-contaminated samples throughout the first three days post metal addition, after which soil respiration became stable (Rajapaksha *et al.*, 2004). However, respiration rates following 10 and 60 days of incubation were not significantly different (Rajapaksha *et al.*, 2004). Lastly, following the addition of lime, a short increase in respiration rates in the contaminated soils was apparent (Rajapaksha *et al.*, 2004). Our data showed no apparent effects of metals on microbial communities since in general limed and unlimed areas have similar levels of total and the bioavailable content (Nkongolo *et al.*, 2013). This suggests that the bioavailable elements in the targeted sites might be too small to be harmful to fungi or bacteria.

Conclusion

The present study reveals that soil liming has significant effect on soil pH and on diversity of fungi species grown on SDA medium. Such difference was not observed when living fungi were considered. PLFA analysis revealed significant differences between limed and unlimed sites for total microbial biomass and fungi. Overall, soil liming affects both fungi and bacteria, but differences in soil respiration appear to be generated by bacterial activities rather than fungi. Overall, our data suggest that soil pH might be the most important factor for microbial abundance and activities than metal content.

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Author's Contributions

Kassandre Goupil: Conducted fungi identification, soil respiration and data analysis and contributed to the manuscript preparation.

Kabwe K. Nkongolo: Coordinated the study, conducted phospholipid fatty acid analyses, integrated all the data and wrote the manuscript.

Sabah Nasserulla: Contributed to fungi identification and data analysis.

Ethics

The authors declare that there is no conflict of interest.

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