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Impact of sub-zero temperatures on the fungal community composition and diversity in short-term petroleum polluted temperate soils

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Abstract

Climate change is already altering the temperate ecosystems and as a result these ecosystems are now subjected to increased incidence of freezing and warming events. To understand the impact of sub-zero temperatures on the fungal communities in temperate soils, three soils namely clean natural soil (CNS), short term diesel oil polluted (SDS) and crude oil polluted (SCS) from a temperate region in Northern China were subjected to freezing and thawing temperatures ranging from –20 to 0°C for 32 days. This study monitors and compares the changes in fungal community composition and diversity in clean and short-term petroleum polluted soils at specific temperature points (20, 0, –10 and –20°C) during the laboratory-controlled freeze-thaw experiments. Structural analysis of the fungal community with 18S RNA gene analysis revealed that the sub-zero temperatures caused distinct shifts in the fungal phylum composition of the temperate soils during soil freezing phase and thawing phase respectively. The community in CNS was most responsive to the sub-zero temperature changes among the studied soil types. The thawing sub-zero temperatures were characterized by decreased community richness in the clean soil while the short-term polluted soils SDS and SCS increased in richness. Overall, our results established that the fungal community composition and diversity in the polluted soil types were more adaptable to the sub-zero temperature variations during soil freezing and thawing conditions when compared to those in the clean natural soil, suggesting that the native fungal communities present in the temperate soils with different contamination profiles displayed varying levels of cold survivability.

Keywords – alpha diversity – freezing temperatures – fungal dynamics – petroleum contamination

Introduction

In recent times, oil spills have become a serious environmental problem in temperate and cold regions such as northern Russia, Canada, Alaska and northern China (Wang et al. 2013, Belkina & Sarkova 2016, Yao et al. 2017). Each year, conservative estimates of around 0.10 to 0.25% of petroleum products pervade the natural environment (Gracia-lor et al. 2011). The environmental impact of contamination is more severe in colder environments as the ecosystems present are generally more sensitive (Gavazov et al. 2017). Petroleum substances have a high potential to accumulate in the soil environment, where they can interfere with the soil microbiome (Nwaichi et

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al. 2015, Wyszkowska et al. 2015). Currently, various physicochemical methods have been employed for the removal and degradation of various recalcitrant petroleum contaminants from soil (Kurniawan et al. 2006). The downside to these methods is that they are often expensive and produce toxic by-products in the process (Khan et al. 2019).

Microorganisms mediated bioremediation is now extensively utilized as an eco-friendly and sustainable alternative for removing the petroleum hydrocarbons in terrestrial and in some cases aquatic environments (Qin et al. 2013). Certain organisms from the fungi kingdom such as white rot fungi have also been considered as attractive options in the remediation of various pollutants (Singh et al. 2015, Kapahi & Sachdeva 2017). Their roles in the degradation of various recalcitrant and persistent pollutants such as dyes, herbicides, heavy metals, aliphatic and aromatic hydrocarbons have been well characterized (Aragão et al. 2019, Akhtar & Mannan 2020). In particular, some species from the fungal genera *Aspergillus*, *Candida* and *Cladosporium* have been shown to be effective in utilizing petroleum hydrocarbon as carbon sources (Silva et al. 2015, El-Hanafy et al. 2017). The ability of these fungi populations to colonize a wide range of heterogeneous environments and adapt to extreme environmental conditions make them attractive candidates for remediating organic contaminants in harsh environments (Bosco & Mollea 2019). Hence, fungal adaptability to extreme conditions in habitat has led to a resurged interest in their identification and isolation from diverse ecosystem including marine (Wang et al. 2018, Vargas-Gastelum et al. 2019) and terrestrial (Dirginciute-Volodkiene & Peciulyte 2011, Thion et al. 2012).

Temperate ecosystems are exposed to sub-zero temperatures at least once a year and periodic soil freeze-thaw cycles (Han et al. 2018). The cold stress associated with such sub-zero temperate range is of ecological interest because of their possible disruptive impacts on soil microbial communities and nutrient transformation in temperate and artic regions (Schimel et al. 2007). Freezing sub-zero temperatures can influence microorganisms directly and indirectly (Clarke et al. 2013). Direct effects include decreased growth rate, enzymatic activities, alteration of cellular composition while indirect effects are observed in the solubility of solute molecules and nutrient diffusion (Tanghe et al. 2003). The role of freezing temperatures on soil properties such as changes in soil structure and chemical properties have been previously examined (Edwards & Cresser 1992, Müller-Lupp & Bölter 2003, Onwuka & Mang 2018), yet not much information is available on the effects of sub-zero temperature gradient on fungal communities present in the soil. Extended cold conditions in soils from temperate regions coupled with other limiting factors such as poor nutrient supply and low moisture content are projected to alter the soil biogeochemical cycling with dramatic effects on soil biology (Yao et al. 2017). Consequently, an in-depth understanding of the diversity and dynamics of fungal communities present in soils periodically subjected to below zero temperatures is required for designing and optimizing effective mycoremediation strategies for petroleum polluted sites situated in cold regions.

Laboratory studies have been greatly helpful in advancing knowledge on the single and multiple biotic and abiotic factors on some soil biological properties (Sándor & Fodor 2012, Xie et al. 2015). However, previous studies conducted on the influence of sub-zero temperature events on microbial communities in pristine and contaminated soils from cold temperate regions (Chang et al. 2011, Yang et al. 2014, Han et al. 2018, Juan et al. 2018, Ren et al. 2018) mostly emphasized on the soil bacterial community. Detailed datasets on the effects of abiotic stress caused by temperatures below zero on fungal communities in petroleum-polluted soils are still lacking. To our knowledge, there are also limited investigations on the combined effects of sub-zero temperature events (abiotic stress) and petroleum contamination (biotic stress) on the fungal community dynamics in temperate soils. We hypothesized that the sub-zero temperature during soil freezing and warming events can have a significant distinct impact on the diversity, abundance, and responsiveness of soil fungal communities, especially in short term petroleum contaminated soils from a cold temperate region. The present research is therefore designed to (1) study the soil fungal community dynamics in mesocosms subjected to laboratory-controlled freeze-thaw events (–20 to 0^{o}C) set at a hypothetical rate of 2^{o}C per day (2) compare differences in abundance and diversity of fungal communities across the soil types during soil freezing and thawing periods.

Materials & Methods

Soil Sampling and Preparation

The methods of sampling, collection and transportation were according to the description of Wu et al. (2016). Clean natural soil (CNS) was collected from an agricultural site in Changchun city, Jilin Province, China (39º7´55´´ N, 117º11´53´´ E), with no history of petroleum contamination and also confirmed by gas chromatography mass spectrometry analysis of C10–C36 petroleum hydrocarbons. The soil samples were sieved through a 2-mm-mesh screen and thoroughly homogenized.

The sampling site is located in a cold temperate continental monsoon climate with an average precipitation of 500–650 nm, an annual average temperature of 1–2ºC, and an average frost-free period of 120–140 days (Guo et al. 2013, Ren et al. 2018).

Preparation of Short-Term Polluted Soils (Soil Amendments)

Crude oil amended soil

Three hundred grams of fresh soil (CNS) was weighed into a 1000 ml glass jar. After that, crude oil was added to the soil at a percentage of 10% (volume/weight) and thoroughly homogenized with a glass rod. This soil was then regarded as the short-term crude oil polluted soil (SCS) in this study.

Diesel oil amended soil

Three hundred grams of CNS was weighed into a 1000ml glass jar. After that, diesel oil was added to the soil at a percentage of 10% (volume/weight) and well mixed with a glass rod. This soil was then regarded as the short-term diesel polluted soil (SDS) in this study. The short-term polluted soils were allowed to stay for two days before the commencement of the freeze-thaw experiment.

Diesel oil (grade B, China National Fuel Standard GB19147–2016) was obtained from Jilin petroleum filling station, Changchun, China. The characteristic parameters of diesel oil are: cetane number – 51, cetane index – 46, density 0.84 $g \cdot cm^{-3}$. The crude oil sample was provided by Sinopec Petrochemical Company, Tianjin China and its density is about 0.91 $g \cdot cm^{-3}$.

Soil Incubation (Sub-Zero Temperature Experiments)

Soil samples were incubated in a specialised temperature and humidity programmable machine (HD-GDJS, China). The samples were incubated for 32 days, starting with a pre-incubation step at +20ºC for 48 hours, then a gradual decrease to –20ºC over 20 days, and a thawing phase with increasing temperature over 10 days back to 0ºC. The freezing and thawing rates were set at 2ºC per day. The samples were obtained at specific intervals (+20, 0, –10, –20ºC) during the experimental freezing phase; these samples will be referred to as $F_{20ºC}$, $F_{0ºC}$, $F_{-10ºC}$, and $F_{-20ºC}$ respectively. Following the freezing phase, samples were obtained at five-day intervals (–10, 0ºC) in the experimental warming phase; these samples will be referred to as $T_{-10ºC}$ and $T_{0ºC}$ respectively. Fig. 1 shows the temperature regime for the study.

At each of the sampling points in the study, two replicates were collected, labelled and immediately stored at –80ºC until DNA extraction.

Soil physicochemical analyses

The initial soil pH was measured by extracting 4 g of soil in 20 mL of 0.01M $CaCl_2.2H_2O$ for 10 min. The calcium chloride extracts were then measured on pH meter (Orion Star A221, Thermo Scientific, USA). Total organic carbon and extractable phosphorus content were determined using the Walkey-Black wet oxidation and Olsen Bicarbonate methods respectively (Walkey & Black, 1934, Olsen et al. 1954). The total petroleum hydrocarbon (TPH) levels in initial soil samples were analysed following a modified version of the method previously described by Buddhadasa et al. (2001). Petroleum hydrocarbon extractions were conducted by sonicating 5 g of each soil sample in

20 ml (Acetone/Methylene chloride (1:1, v/v) solvent) for 75 mins and TPH concentration was measured in 1 ml aliquots using Gas Chromatography-Mass Spectrometry (Agilent 6890). Chromatographic separation was achieved using HP5-MS (30 m × 0.32 mm I.D. and 0.25 pm F.T.) column and coupled to a HP 5970 MSD. Injected sample volume was 1 µl. Helium was used as the carrier gas (0.98 ml min^{-1}). Samples injection mode was splitless. Injector temperature and detector temperature were set at 280ºC and 290ºC respectively. Oven temperature was programmed from 60 to 240ºC (5 min hold), at 6ºC min^{-1}, and from 240 to 300ºC (15 min hold), at 6ºC min^{-1} rate.

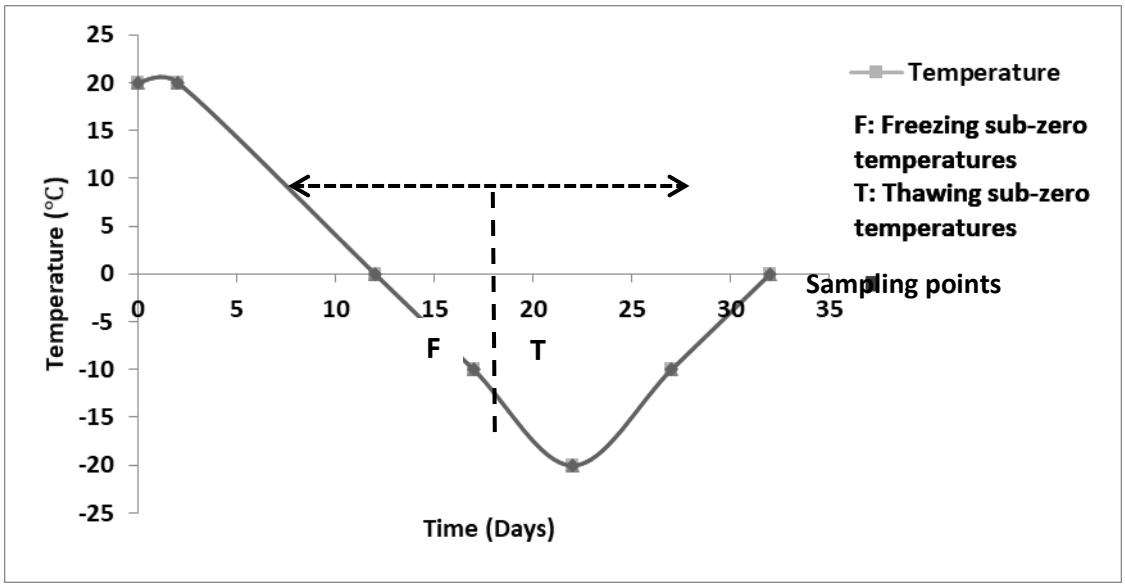

Fig. 1 – Temperature regime in the study.

DNA extraction, amplification and sequencing

Soil DNA was extracted by OMEGA E.Z.N.A™ Mag-Bind Soil DNA kit (Omega Bio-tek, Inc, GA, USA) according to manufacturer's instruction. The DNA samples were labelled F1 to F40 (Table 1). DNA quality was assayed on 1% agarose gel and the quantity of DNA was determined by Qubit 3.0 DNA detection kit (Invitrogen, Carlsbad, CA, USA). A total of 10–20 ng of DNA was used to generate amplicons using MetaVx™Library Preparation kit (GENEWIZ, Inc, NJ, USA). The DNA was amplified by PCR using a set primer of the 18S rRNA ribosomal gene that target highly conserved regions NS1 (5'-GTAGTCATATGCTTGTCTC-3') and GC_fung (5'-GCATTCCCCGTTACCCGTTG-3') (White et al. 1990, Cisneros-de la Cueva et al. 2016). PCR procedures including the reaction mixture and thermal profile were conducted as previously described by Li et al. (2016). After successful amplifications, the PCR products were purified with the QIAquick PCR Purification Kit (Qiagen) and quantified using the PicoGreen dsDNA Assay kit (Invitogen). Next-generation sequencing was conducted on an Illumina Miseq Platform (Illumina, San-Diego, USA) at Sangon Biotech Co., Ltd., Shanghai, China.

Statistical and Bioinformatics analyses

All merged raw sequences were trimmed using the Quantitative Insights into Microbial Ecology (QIIME) toolkit v.1.7.0 (Caporaso et al. 2010). After ambiguous bases containing "N" were trimmed, joined sequences with lengths between 240 and 260 base pairs were subjected to chimera removal by U-Chime (Edgar et al. 2011) in Mothur (v.1.30.1). Lastly, OTU clustering was performed using UCLUST at a similarity level of 97% (Edgar 2010), and taxonomic groups were assigned using Ribosomal Database Project Classifier with a minimal 80% confidence estimate (Quast et al. 2013). The rarefaction curve plots and Venn diagram highlighting unique OTUs for the fungal communities were produced using the R software (R Core Team 2013). The Mothur software (version v.1.30.1) was used to estimate coverage according to Good's estimator; and the

community richness and diversity were estimated using the Chao index, abundance coverage estimator (ACE), and Shannon index.

Results

Initial soil physicochemical properties

The results showed that the soil samples (CNS, SDS and SCS) consisted of fine grain particles (Table 1). Overall in the present study, the soils all had low moisture content (moisture content below 5%) and initial pH values in all the soil groups ranged from 7.32–7.84 The TPH concentrations in the soils was in the following order SDS>SCS>CNS. However, SDS had the highest total carbon and organic matter content compared to the other soil groups. Generally, the total carbon and organic matter contents were higher in the contaminated soil groups (SCS and SDS).

Table 1 Some physicochemical parameters of the three soils used for the study*

Parameters	CNS	SCS	SDS
Soil Type	fine grain	Fine grain	Fine grain
Gravimetric soil moisture (%)	3.15 ± 0.02	4.20 ± 0.04	4.55 ± 0.06
Soil pH (as $CaCl_2$ extract)	7.50 ± 0.04	7.84 ± 0.00	7.32 ± 0.01
Total Petroleum Hydrocarbons C_{10}–C_{36}(mg/kg)	ND	3274.86 ± 405.03	6960.46 ± 467.42
Total Organic Carbon (%)	1.30 ± 0.35	1.40 ± 0.14	1.80 ± 0.28
Soil Available Phosphorus (mg/kg)	32.56±1.34	20.25±0.84	23.04±0.99
Soil Organic Matter (%)	2.21 ± 0.60	2.38 ± 0.24	3.06 ± 0.40

Values here are expressed as mean ± SD
ND = total petroleum hydrocarbon level < 5mg/kg
CNS = Clean natural soil, SDS- Short term diesel polluted soil
SCS = Short term crude oil polluted soil

Sequence analysis

A total of 1289785 sequences with an average read length of 260 bp were generated for the three soil samples studied at different incubation temperatures. After quality control, a total of 839645 sequences (65.1%) were used for the analysis. The numbers of sequences for each are listed in Table 2. The clustering of normalized sequences at 97% similarity resulted in 13858 OTUs. Good's coverage was higher than 99.9% (Table 2) throughout the samples. This data indicates an excellent overall OTU coverage afforded by the deep sequencing.

Table 2 Details of the obtained sequence reads, fungal OTU richness, coverage, and diversity indices in each sample during different incubation temperatures

Temp (°C)	Sample	Sequence reads	Number of OTUs	Good Coverage	Shannon	Simpson index	ACE	Chao
F 20	CNS	26749	548	0.99	2.48	0.21	790.83	745.81
	SDS	29567	579	0.99	3.72	0.08	729.51	748.03
	SCS	43732	223	0.99	0.28	0.94	364.43	353.63
F 0	CNS	45763	782	0.99	3.58	0.09	979.93	967.4
	SDS	20597	450	0.99	3.59	0.07	542.52	552.24
	SCS	44395	213	0.99	0.36	0.91	383.04	333.68
F–10	CNS	30034	518	0.99	2.16	0.33	659.82	642.03
	SDS	29645	694	0.99	4.06	0.07	821.81	820.97
	SCS	62998	275	0.99	0.68	0.82	343.18	345.66
F–20	CNS	36887	675	0.99	3.71	0.06	874.87	851.17
	SDS	42881	555	0.99	3.80	0.06	723.30	748.40
	SCS	35360	323	0.99	0.72	0.81	412.70	428.30

Table 2 Continued.

Temp (°C)	Sample	Sequence reads	Number of OTUs	Good Coverage	Shannon	Simpson index	ACE	Chao
T–10	CNS	39879	569	0.99	3.75	0.06	711.98	714.32
	SDS	59921	326	0.99	3.53	0.06	542.89	430.43
	SCS	47765	243	0.99	1.57	0.51	314.72	312.19
T 0	CNS	37841	516	0.99	1.82	0.48	621.03	590.54
	SDS	49406	434	0.99	3.38	0.06	695.82	584.63
	SCS	79206	352	0.99	1.01	0.51	433.66	427.83

Abbreviations: CNS = Clean natural soil, SDS = Short term diesel polluted soil, SCS = Short term crude oil polluted soil

Fungal diversity and communities

The observed OTUs number in each sample at temperatures 20, 0, –10 and –20°C are shown in Table 2. At the beginning of the study (F 20°C), the highest number of OTUs was obtained in short-term diesel polluted soil SDS, followed by clean soil CNS (548 OTUs), and short-term crude oil polluted soil SCS (223 OTUs). SCS had the lowest number of OTUs (213–352 OTUs) during the sub-zero temperatures (0, –10 and –20°C). The Shannon and Simpson diversity index showed that the highest fungal diversity was observed in SDS. Fungal diversity however increased in SDS and SCS during the sub-zero temperatures. Specifically, the percentage increase in Shannon diversity indices in SDS and SCS were 5.53 and 48.61% respectively as the incubation temperature decreased from 0°C to –20°C. In the CNS, fungal diversity decreased by 39.66% as the incubation temperature decreased to –10°C.

At the beginning of the study (F 20°C), a total of 1350 OTUs were recovered from the clean and short-term polluted soil samples. The composition of the 548 OTUs at the phylum level in the clean soil CNS was assigned to Basidiomycota (66.86%), Ascomycota (29.80%), unidentified fungi (0.06%), unclassified Eukaryota (2.79%) Fig. 2a. The phylum composition in the short-term polluted soils SCS and SDS were different from the clean soil. The OTUs in SDS was assigned to Ascomycota (68.32%), Basidiomycota (12.27%), unidentified fungi (0.03%) and unclassified fungi (2.91%) Fig. 2b while the OTUs in SCS were assigned majorly to unclassified fungi (97.86%) Fig. 2c.

The gradual decrease from F 20°C to 0°C (Fig. 3) and then to the first sub-zero incubation temperature F –10°C (at the rate of 2°C per day) resulted in notable shifts in the fungal phylum composition in the three soil samples. Ascomycota accounted for more than 7% of the fungal community in CNS (Fig. 4a) and was 2.57 times higher than its initial value at the beginning of the study (20°C). Similarly, at the sub-zero incubation temperature (F–10°C) Ascomycota in SDS (73.2%) and SCS (4.65) (Fig. 4b, c) increased in relative abundance compared to their respective abundance at 20°C.

By the second sub-zero temperature (F–20°C), the relative abundance of fungi belonging to Ascomycota varied in the three soil samples – the abundance decreased in CNS, increased in SCS and remain unchanged in SDS (Fig. 5a, b, c). We further examined the influence of sub-zero temperatures on fungal community by increasing incubation temperature from F –20 to T –10°C and then to T 0oC. The predominant fungal phylum in the clean soil CNS shifted from Ascomycota (T–10°C) to Basidiomycota (T 0°C) while in SDS, Ascomycota remained the predominant phylum at T–10°C and T 0°C respectively (Figs 6, 7a, b, c).

At the class level, the assignable fungal OTUs at the beginning study were dominated by Agaricomycetes (64.60%) in CNS, Dothideomycetes (47.48 %) in SDS and unclassified fungi (97.86) in SCS (Fig 8a). At 0°C, Dothideomycetes was the predominant class in CNS and SCS (Fig. 8b). By F –10°C, the dominant fungal class in the soil shifted to those from Saccharomycetes (56.53 %) in CNS while Dothideomycetes and unclassified fungi remained dominant in SDS and SCS respectively (Fig. 8c). Dothideomycetes dominated in CNS and SDS during the second sub-zero incubation temperature, F–20°C (Fig. 8d). Reversing the incubation temperature from the sub-

zero temperature F–20°C to T 0°C resulted in a shift dominant fungal class population in the clean soil. Agaricomycetes (71.56%), followed by Saccharomycetes (11.43%) dominated in CNS while by Dothideomycetes and unclassified fungi remained dominant in SDS and SCS respectively the end of the study (Fig. 8e, f).

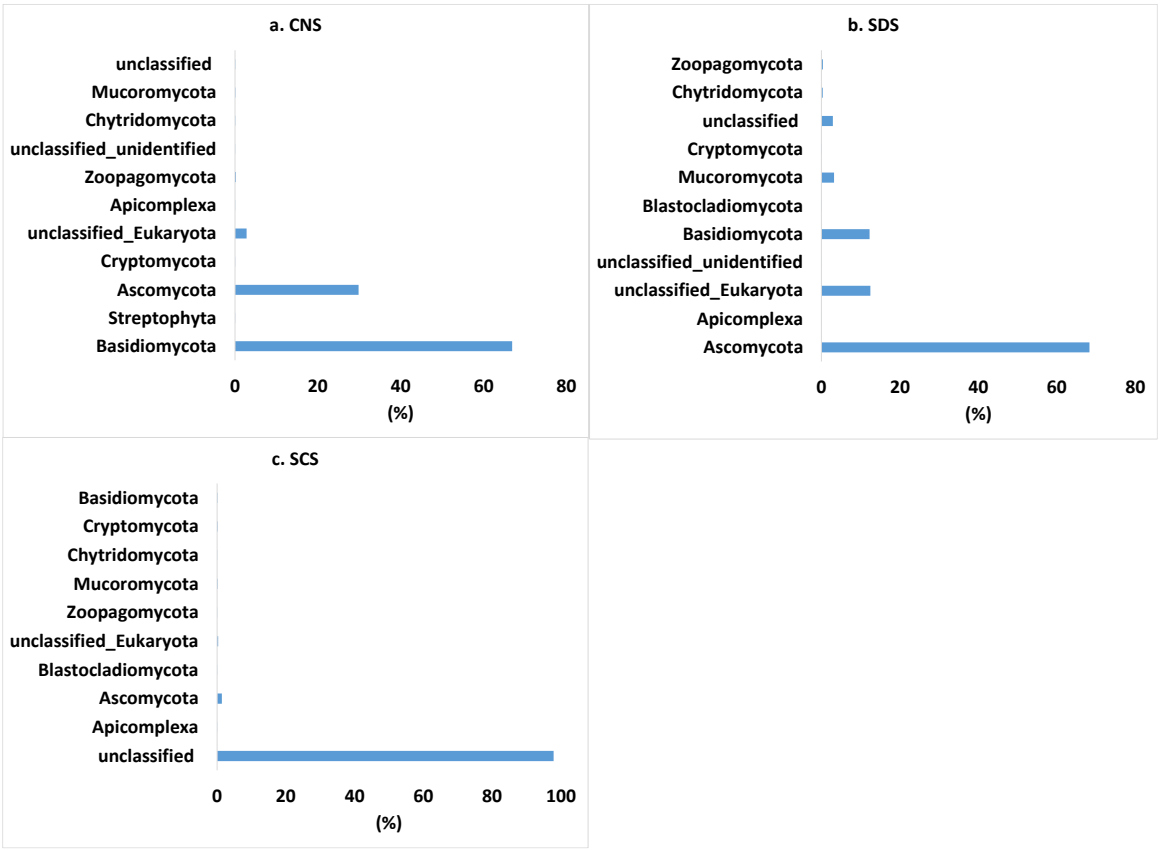

Fig. 2 – Phylum composition. a Clean Natural Soil, CNS. b Short term Diesel Polluted Soil, SDS. c Short term Crude oil Polluted Soil, SCS at F 20°C.

The most frequently detected fungal genera in the clean natural soil (CNS) were *Sanghuangporus* (62.80%) and *Didymella* (12.06%) at F 20°C. Less abundant genera (< 1% of average relative abundance) such as *Penicillium* (0.44%), *Epicoccum* (0.58%), and *Saccharomyces* (0.77%) were also initially present in the CNS (Supplementary Fig. 1a). In contrast, the short-term polluted soil SDS was dominated by *Didymella* (23.47%) and *Cladosporium* (15.87%) whereas unclassified fungi (97.68%), *Pseudogymnoascus* (0.55%) and *Cladosporium* (0.05%) were the dominant genera in the short-term crude oil polluted soil SCS (Supplementary Fig. 1b, c). The first sub-zero temperature F-10°C (Supplementary Figs 3a, b, c) was characterized by changes in the relative abundance and distribution of the genera in the clean soil and contaminated soil. *Saccharomyces* (53.67%) increased while *Sanghuangporous* (15.44%) decreased when compared to its abundance at F 20°C. On the other hand, *Cladosporium* in SDS increased by 3% while *Pseudogymoascus* similarly increased by 1.25% in SCS at F –10°C (Supplementary Fig. 3b, c). By the second sub-zero temperature F –20°C, we observed another shift in the distribution of the dominant genera in the clean soil. *Saccharomyces* decreased from 53.67% to 5.32% while *Sanghuangporous* (14.45%) became dominant in CNS (Fig. S4 a). *Epicoccum* significantly increased from 5.56% (F –10°C) to 14.93% (F –20°C) making it the second most abundant fungal genera in SDS (Supplementary Fig. 4b). *Pseudogymoascus* in SCS was 1.61 times higher by F-20°C (Supplementary Fig. 4c) when compared to the second sub-zero temperature F –10°C (Supplementary Fig. 3c). By the end of the study, increasing the incubation temperature gradually to T 0°C led to the following changes: *Sanghuangporous* increased to 70.78%, followed by

appearance of *Hanseniaspora* (10.69%) in CNS (Supplementary Figs 5a, 6a), *Didymella* decreased from 23.47 to 12.97% in SDS (Supplementary Figs 5b, 6b) while *Pseudogymnoascus* increased from 0.55 to 3.31% in SCS (Supplementary Figs 5c, 6c).

As seen in Supplementary Figs 7–12, the composition of fungal communities at the species levels in the three soil samples showed different distribution pattern, the relative abundance of each fungal taxonomic group also showed significant difference in each soil sample at the various incubation temperatures. The NMDS ordination of fungal community structure (abundance-based metric) showed that there was low similarity between the clean soil sample and the short-term crude oil polluted soil during the sub-zero temperatures while there was slight level of inclination between CNS and SDS during the same incubation temperature regimes (Fig. 9).

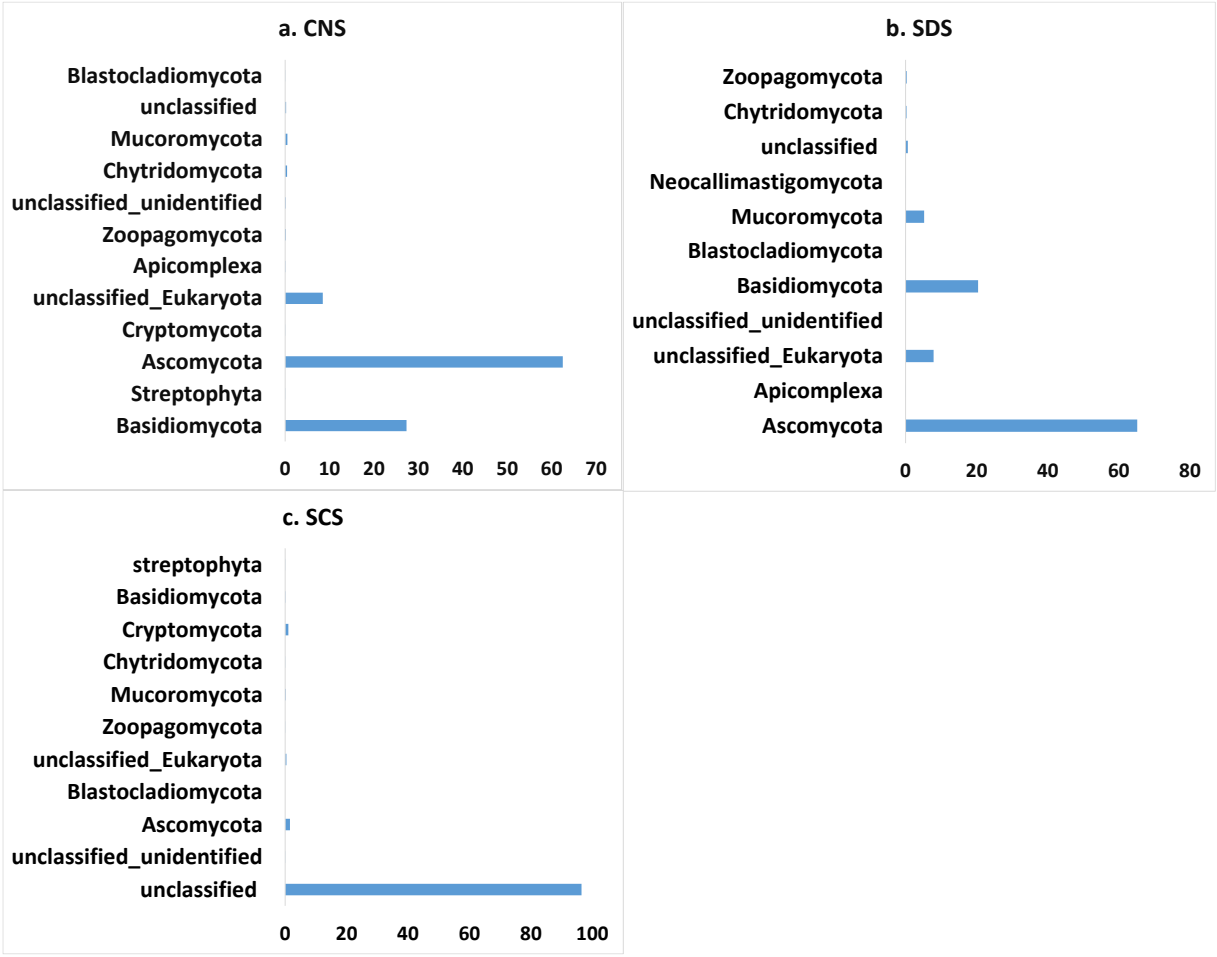

Fig. 3 – Phylum composition. a Clean Natural Soil, CNS. b Short term Diesel Polluted Soil, SDS. c Short term Crude oil Polluted Soil, SCS at F 0°C.

Discussion

Initial Soil Chemical Properties

The particle size distribution in a soil is an important property that influences soil water retention, trafficability and infiltration rate (Ukah et al. 2019). Sandy soils also tend have higher permeability compared to clay soils (Delgado & Gomez 2016) suggesting that the soils may show lower permeability during the experiment. The soils also had low moisture content. Wang et al. (2013) had previously reported that oil pollution could significantly lower soil moisture content. Our results were in accordance with their findings. The initial pH values showed that the soils were all slightly alkaline in nature. This agrees with previous reports of alkalescency in soils from cold temperate regions (Smith et al. 2002). Furthermore, previous studies on petroleum-polluted sites in

China showed that oil pollution raised soil pH, ion exchangeable acidity and effective cation exchange capacity (ECEC) in soils. Generally, the total carbon and organic matter contents were higher in the contaminated soil groups (SCS and SDS). Naga Raju et al. (2016) similarly reported higher organic matter content in polluted soils compared to control soil. The higher total carbon and organic matter in the contaminated soil groups may largely be due to the introduction of hydrocarbon contaminants that are mostly organic in nature.

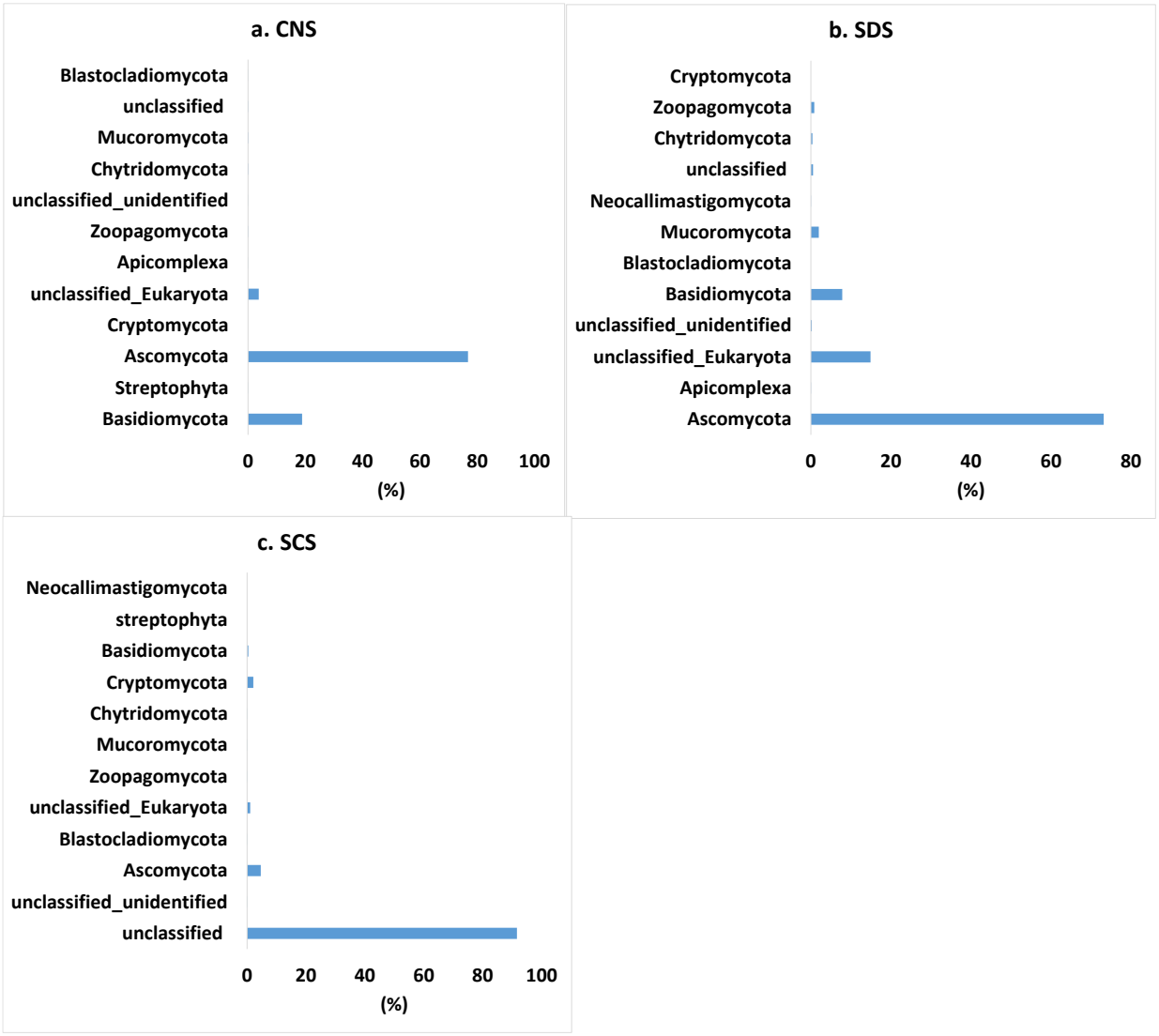

Fig. 4 – Phylum composition. a Clean Natural Soil, CNS. b Short term Diesel Polluted Soil, SDS. c Short term Crude oil Polluted Soil, SCS at F –10ºC.

Effect of petroleum contamination on the fungal community composition and diversity in the temperate soils

The beginning of the study (F 20℃) provided an ideal opportunity to evaluate the impact of short-term biotic stress caused by the introduction of petroleum oil on the soil fungal communities before subjecting the soils to sub-zero incubation temperatures. The characteristics of the fungal community varied in the three soil groups with hydrocarbonoclastic fungal genera such as *Cladosporium*, *Pseudogymnoascus*, *Aspergillus* and *Penicillium* being selectively enriched in the short-term petroleum polluted soils SCS and SDS. The results correspond roughly with previous reports. Ameen et al. (2016) identified *Cladosporium* as one of the main fungal genera with great diesel oil degrading potential in mangrove sediments while the abilities of *Pseudogymnoascus*, *Aspergillus* and *Penicillium* to utilize aliphatic and aromatic compounds have also been previously

established (Sutton et al. 2013, Borowik et al. 2017, Prenafeta-Boldú et al. 2019). In our study, we also observed a notable shift in the dominant phylum from Basidiomycota in CNS to Ascomycota and unclassified fungi in SDS and SCS respectively. The introduction of diesel and crude oil respectively led to the appearance of some distinctive fungal populations such as *Mortierella*, *Fusarium*, *Cenococcum Thyridium* and *Valsa* that were not found in the clean soil. Most of the fungal populations that appeared in the soil contaminated with crude oil SCS were unidentified. Therefore, further study may be required to identify these highly novel fungi as possible suitable candidates for mycoremediation. With respect to the richness of fungal diversity in the soils at the beginning of the study, SDS had the highest diversity. Data from our initial physicochemical properties of the soils highlighted that SDS also had highest amount of total organic carbon. Tedersoo et al. (2014) had previously reported a strong correlation between total organic carbon and richness of fungal diversity and their observation was consistent with the results from this study. From our results, we infer that the introduction diesel and crude-oil caused distinct shifts in fungal community composition and diversity in the soils. Hence, the nature of contaminant present in the soil remains an important factor that controls fungal community dynamics in polluted soils.

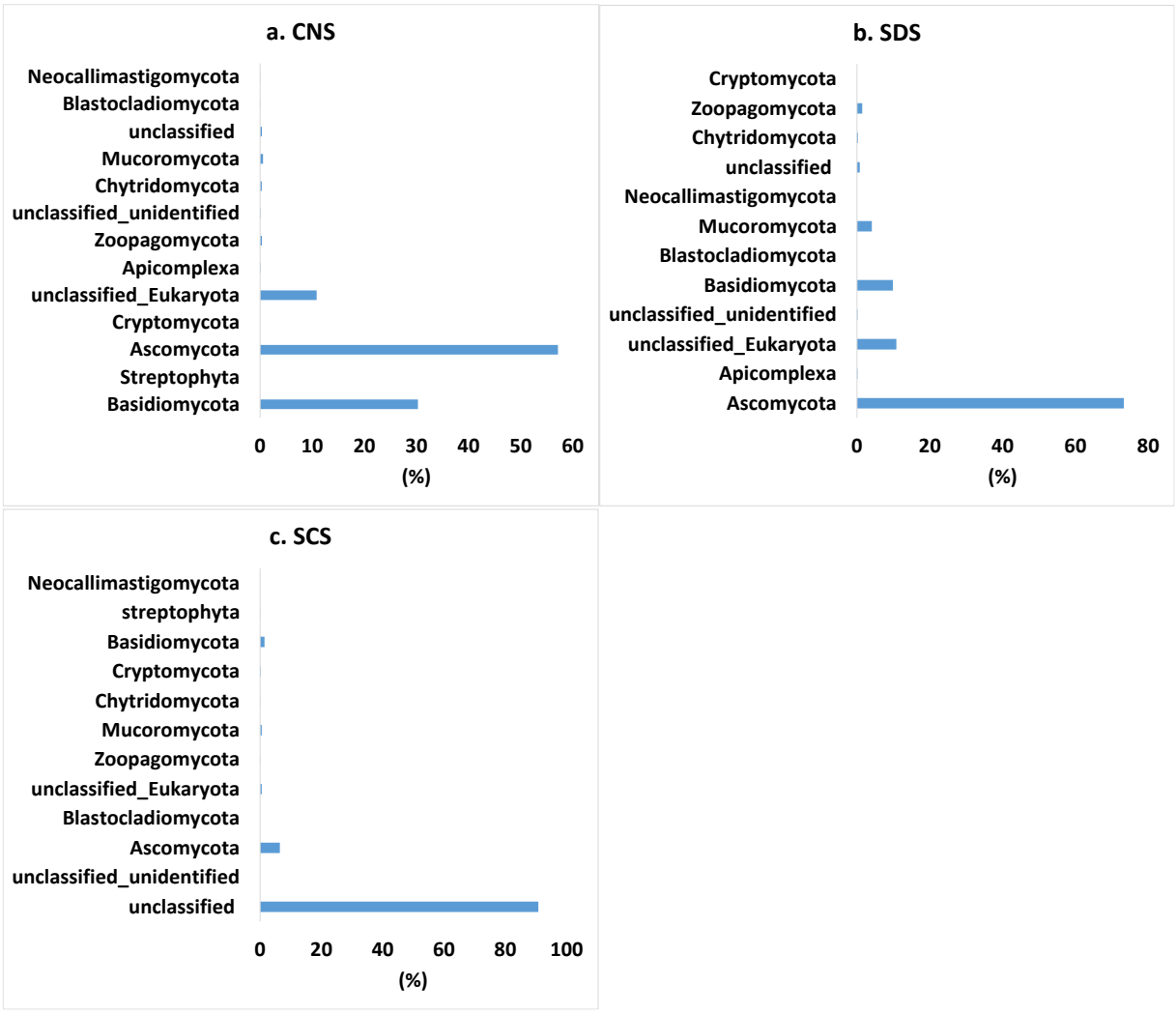

Fig. 5 – Phylum composition. a Clean Natural Soil, CNS. b Short term Diesel Polluted Soil, SDS. c Short term Crude oil Polluted Soil, SCS at F –20ºC.

Impact of sub-zero temperatures on the characteristics of the fungal community in clean and short-term petroleum polluted soils

The phylogenetic composition and diversity of the fungal communities present in the clean and short-term petroleum polluted soil samples at different sub-zero incubation temperatures were

profiled using pyrosequencing PCR-amplified fungal 18S rRNA gene fragments. In particular, we monitored how the indigenous fungal communities in the newly contaminated soils responded at different temperatures points below 0°C. The relative abundances of the predominant fungal phylum in the soils responded differently to the sub-zero incubation temperatures. This sub-zero temperature sensitivity trend was most pronounced in the clean natural soil CNS. The order of freezing temperature tolerance of the dominant fungal genera in the soils was SCS>SDS>CNS. One possible explanation for this variation observed in the different soil types exposed to the same freezing sub-zero temperature regime in this study is increased levels of substrate availability in the polluted soils (Onwuka & Mang 2018).

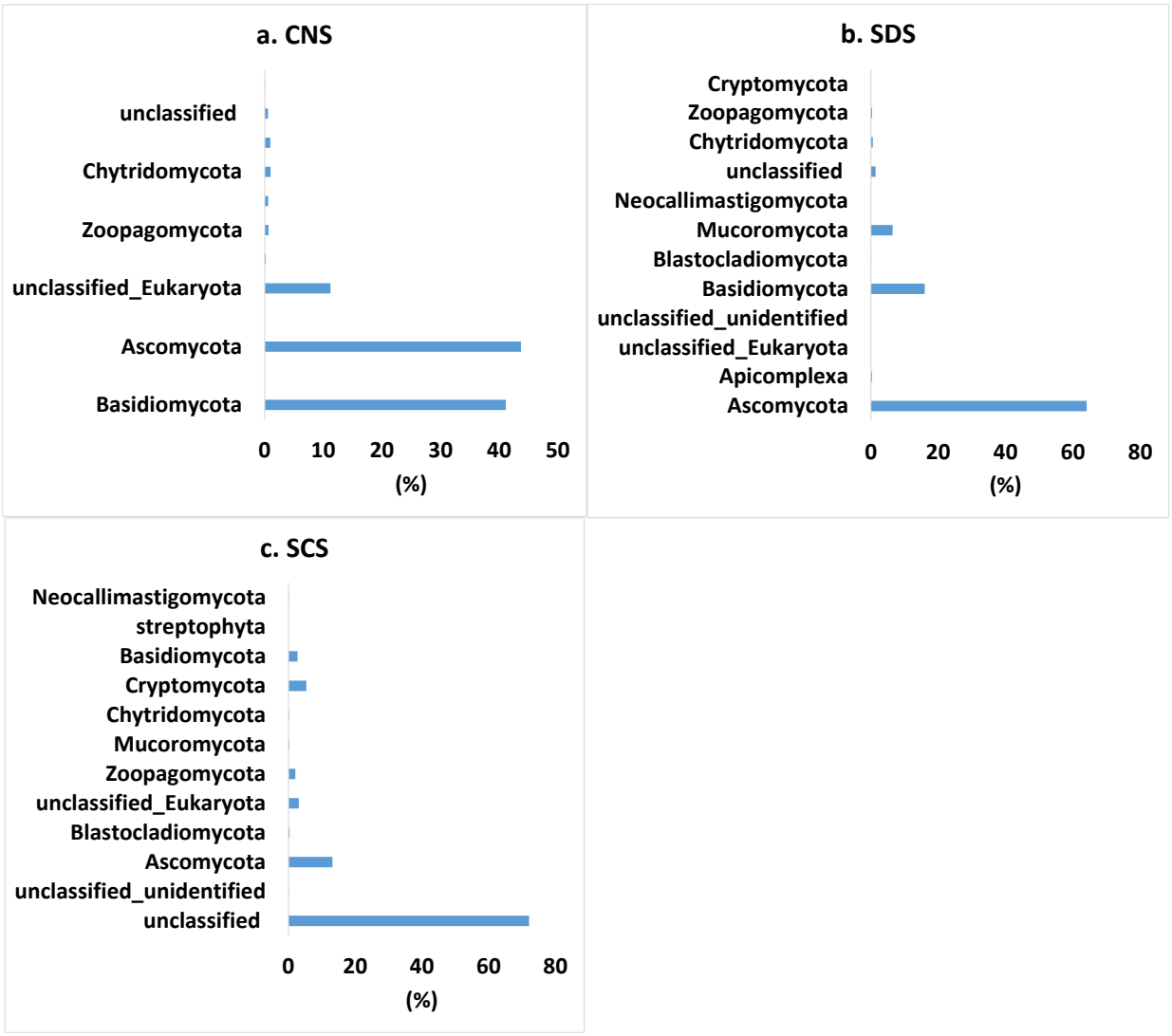

Fig. 6 – Phylum composition. a Clean Natural Soil, CNS. b Short term Diesel Polluted Soil, SDS. c Short term Crude oil Polluted Soil, SCS at T –10°C.

Freezing sub-zero temperatures (< 0°C) have been shown to generally inhibit microbial growth (Allison & Treseder 2008). This was the similar case for the clean soil CNS in our study, the initial dominant phylum Basidiomycota decreased to one-half of its initial value (F20°C) when incubation temperature decreased to –10 and –20°C respectively. In contrast, we observed that the dominant phylum Ascomycota in the short-term petroleum polluted soils SDS and SCS displayed physiological adaptability to lower temperatures (F–10 and F–20°C). These adaptive behaviours may also be attributed to ability of the predominant genera in the polluted soils to utilize petroleum hydrocarbons and retain metabolic activities for growth and survival during the soil-freezing phase. Wu et al. (2018) also identified Ascomycota as one of the dominant fungal group in both

contaminated and low contaminated soils in their study. The members of Ascomycota are known to fulfil essential roles as decomposers of complex organic molecules in both terrestrial and aquatic habitats (Perkins et al. 2019). The relative abundance of Ascomycota varied substantially with soil contamination profile and freezing sub-zero temperatures (F 0°C, F–10, F–20°C) in this study.

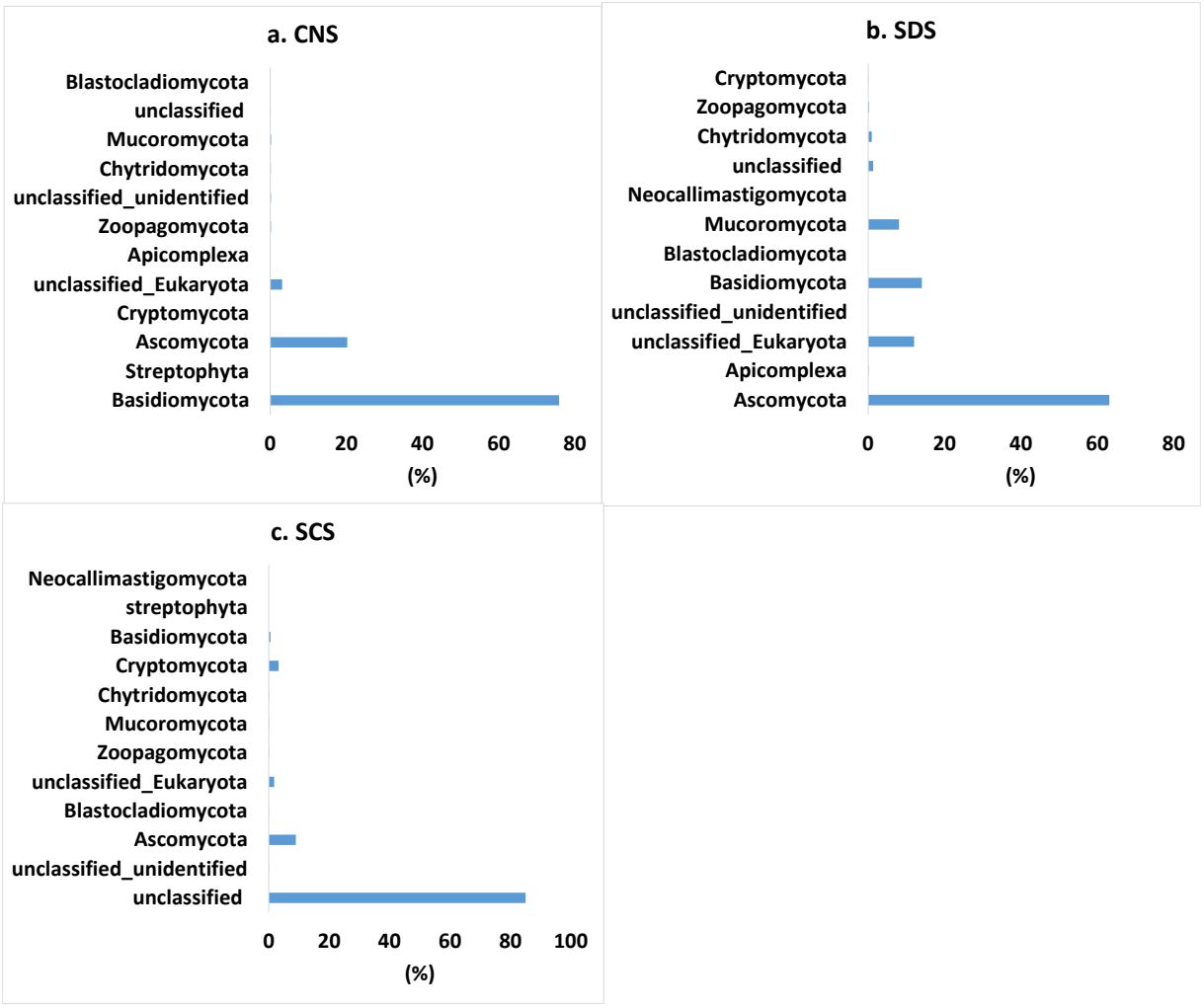

Fig. 7 – Phylum composition. a Clean Natural Soil, CNS. b Short term Diesel Polluted Soil, SDS. c Short term Crude oil Polluted Soil, SCS at T 0°C.

The impact of sub-zero temperatures on the soil fungal community composition was more evident in the clean soil at the class level compared to the short-term polluted soils. For each ten degree decrease in temperature, there was a corresponding redistribution of the fungal class population in CNS- we observed a shift from Agaricomycetes to Saccharomycetes and then to Dothideomycetes while in SDS and SCS, Dothideomycetes remained as the dominant identified class population throughout the sub-zero incubation temperatures (F 0, F–10 and F–20°C rant of the sub-zero temperatures. This sub-zero temperature tolerance effect was further confirmed by the values of the Shannon diversity index in the soil samples during F–10°C where fungal diversity in the short-term polluted soils increased by 8.37% and 58.82% in SDS and SCS respectively. Previous studies have reported the temperature tolerance of some fungi isolated from cold temperate region (Li et al. 2012, Wang et al. 2015). Our results were consistent with previous findings.

Impact of soil thawing on the fungal communities in the clean and polluted soils

Most fungi isolated from the cold temperate environments have been described as cosmopolitan i.e. they have wide growth temperature ranges (Wang et al. 2017). In the present

study, we also attempted to monitor the effect of sub-zero temperature changes on the indigenous fungal species present in the clean and polluted during periods of soil thawing by gradually increasing the temperature from –20 to 0ºC. The results obtained during T–10ºC and T 0ºC revealed that the composition of active fungal species in the clean soil during soil thawing differed from what we earlier observed during the soil freezing period. Some fungal species like *Albugo laibachi* and *Hanserniaspora uvarium* appeared in the clean soil as temperature gradually rose while others like *Sanghuangporous baumii* and *Sugiyamella xylanicola* increased in abundance. Rillig et al. (2016) earlier reported that soil warming increased the abundance of arbuscular mycorrhizal fungi in annual grassland. Also, in a previous study by Gange et al. (2007), they observed that increasing temperatures resulted in increased abundance of fungal strains in the soil. Although these prior studies were conducted on clean soils, our results for the short-term polluted soils SDS and SCS are consistent with their reports on the positive effects of soil warming on fungal communities.

The results obtained during the soil-thawing period of the study were very interesting. It suggested that although the sampling temperature points (0, –10ºC) were the same as the freezing phase, the fungal communities in both clean and short-term polluted soils were distributed differently during soil freezing and thawing phases. This may be explained by the increased availability of water (moisture content) that occurs as the soil transition from freezing to thawing temperatures. Fungi are very successful inhabitants of soil due to their high capacity to adopt various forms in response to adverse or unfavourable conditions (Sun et al. 2005). NMDS ordination data from this study illustrated that fungal community structure in the different soil groups underwent significant shifts in response to the warming temperatures with the relative abundance of the predominant genera in the soils showing distinct variations in percentage. However, majority of the fungal species that occurred in the short-term crude oil polluted soils during the thawing phase of the study were unidentified further investigation will be needed to identify these fungal species and their ecological roles.

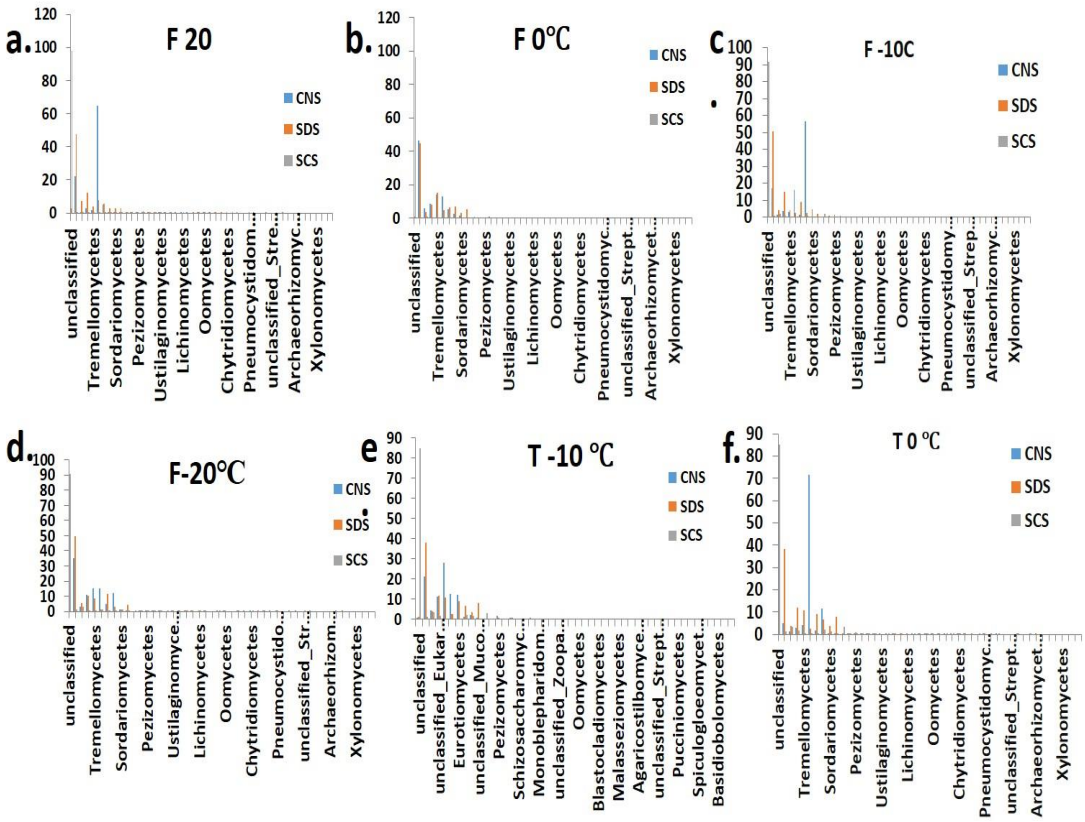

Fig. 8 – a-f Abundance of detected fungal sequences at the class level in each sample at (A). F20ºC (B). F 0ºC (C). F –10ºC (D). F –20ºC (E). T –10ºC (F). T 0ºC

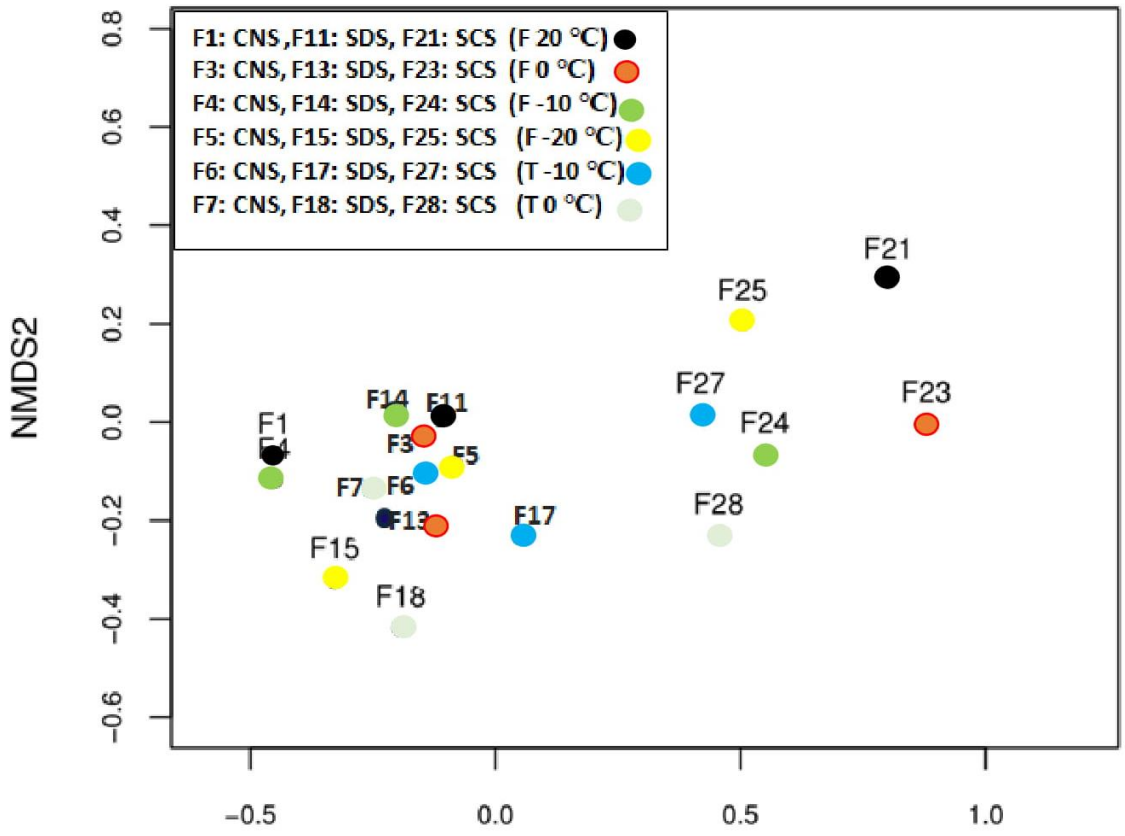

Fig. 9 – NMDS ordination of the fungal community structure. Filled symbols represent clean and contaminated soil samples. The alphabetical letters and numbers beside the symbols represent the sample collected at the different incubation temperature (F 20°C, F 0°C, F –10°C, F –20°C, T –10°C, T 0°C). Presence-absence based, 2D stress was 0.03.

Conclusion

This study comparatively assessed the effects of experimental sub-zero temperatures on the distribution characteristics and diversity of fungal communities in clean (pristine), short term diesel- and crude oil-polluted soils collected from a cold temperate region. We found that although the experimental freezing and warming sub-zero temperatures played a significant role in the redistribution characteristic of the soil fungal communities, the incidence of petroleum pollution still remains a crucial factor that can directly affect fungal community structure and diversity in temperate soils. Our results indicated that fungal diversity and richness in the temperate soils varied differently in response to the experimental freezing and thawing temperatures in the study with higher effects being observed in the fungal communities of the clean natural soil compared to the short-term petroleum polluted soils. In general, our results contribute to predictive understanding of fungal community dynamics in response to temperature variation (abiotic stressor) and have important implications for the mycoremediation of short-term petroleum polluted soils from temperate regions.

Ethics approval and Consent to participate

Not applicable as the present study does not involve human subjects.

Availability of data and materials

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Conflicts of Interests

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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Authors' contributions

Conceptualization: [Liu Na], [Chioma J. Okonkwo]; Methodology: [Jialu Li], [Chioma J. Okonkwo] Formal analysis and investigation: [Chioma J. Okonkwo], [Jialu Li]; Writing – original draft preparation: [Chioma J. Okonkwo]; Writing – review and editing: [Liu Na], [Chioma. J. Okonkwo]; Funding acquisition: [Liu Na]; Supervision: [Liu Na]

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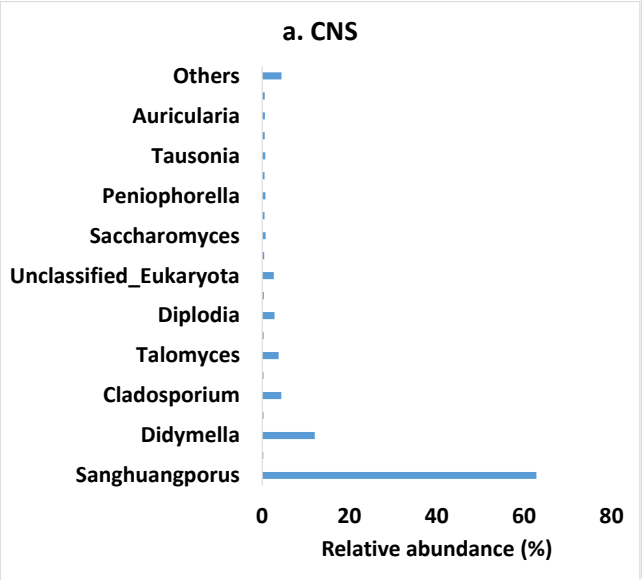
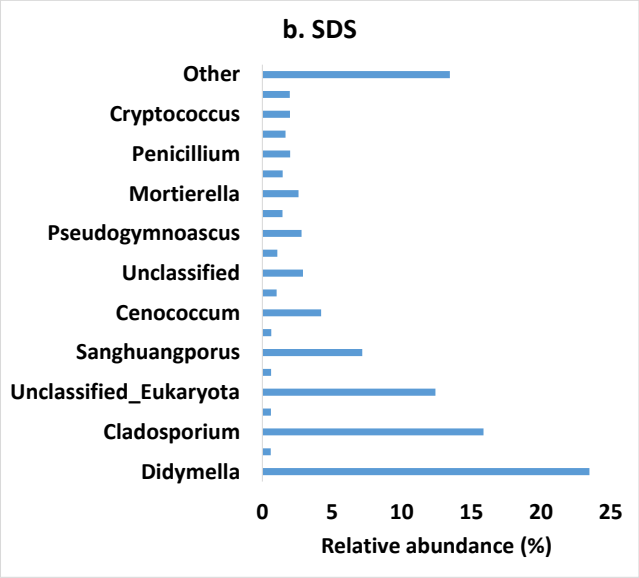
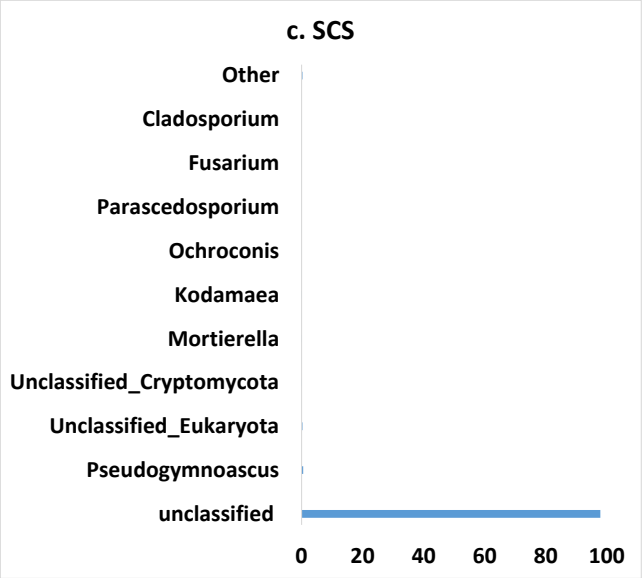
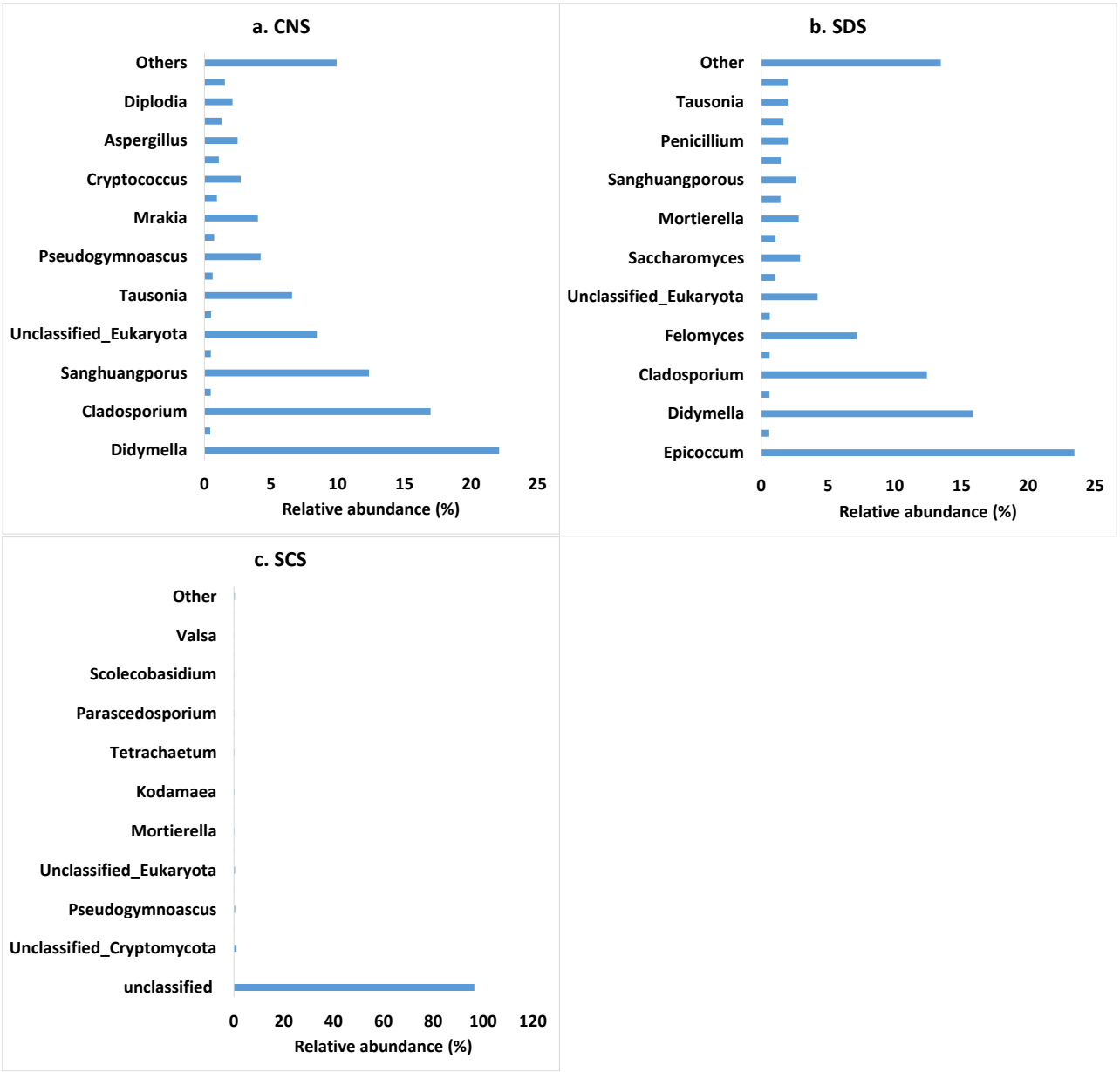


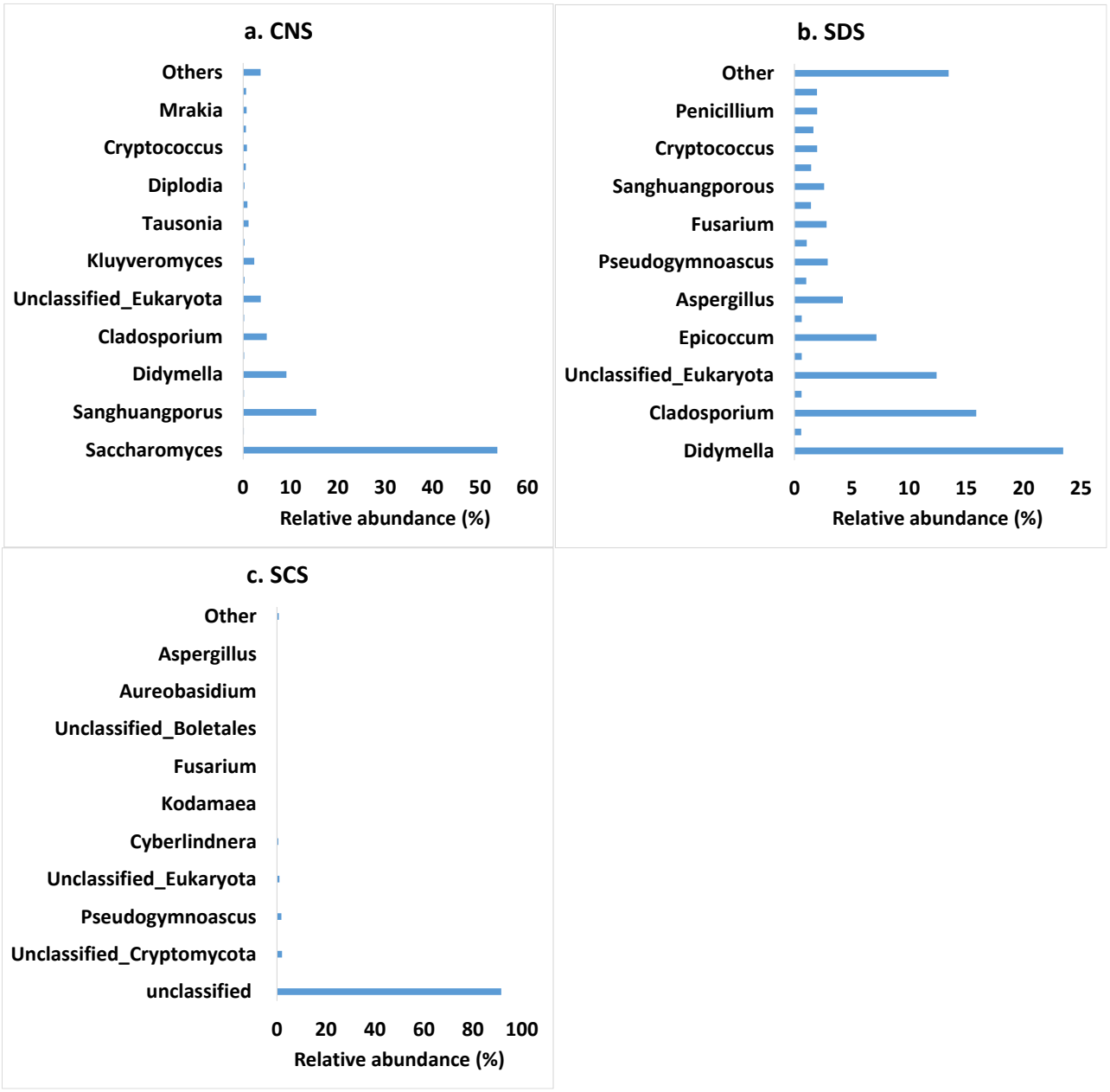




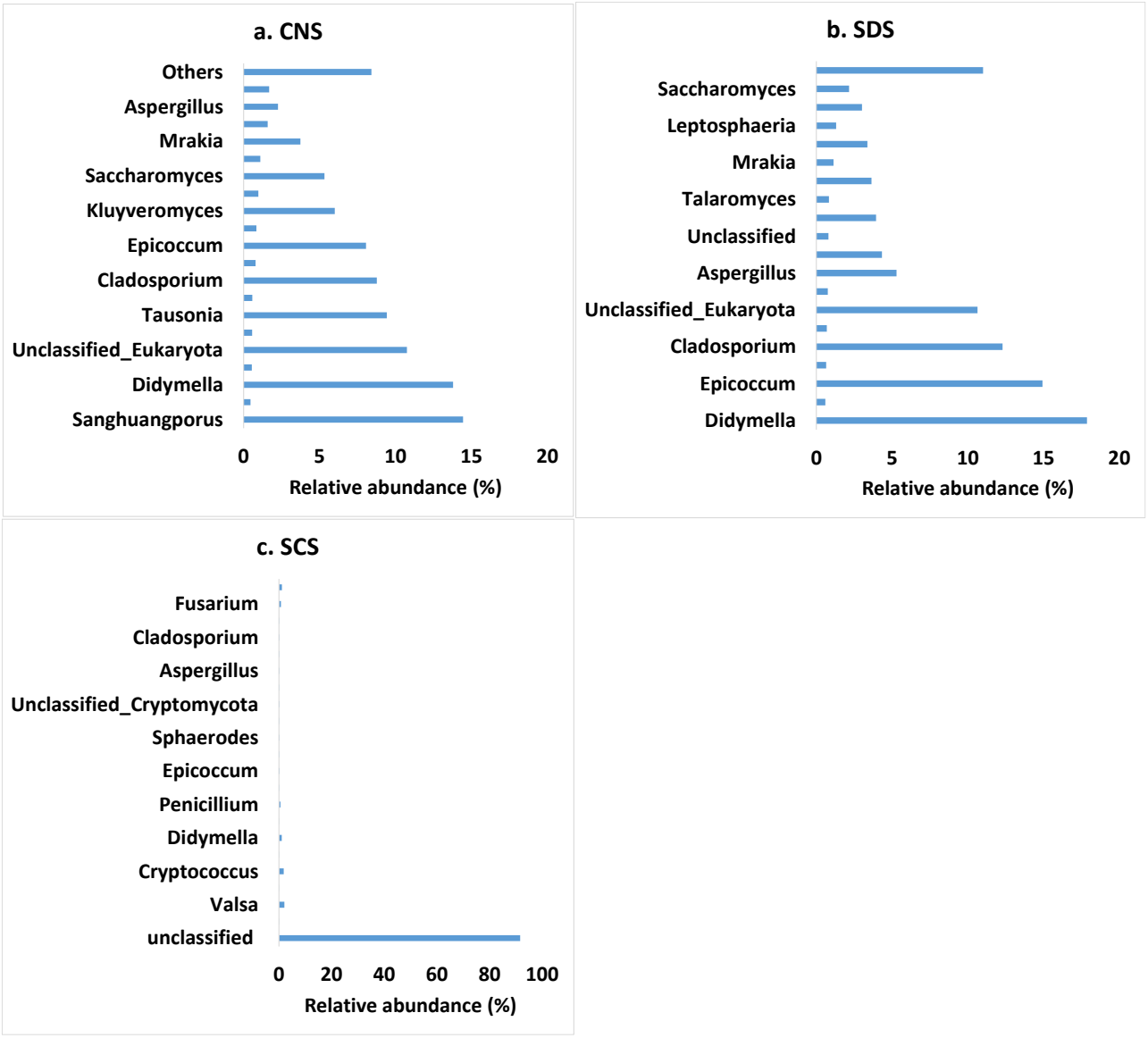
Supplementary Fig. 1 – Genus composition of (a) Clean Natural Soil, CNS (b). Short term Diesel Polluted Soil, SDS (c). Short term Crude oil Polluted Soil, SCS at F 20°C.



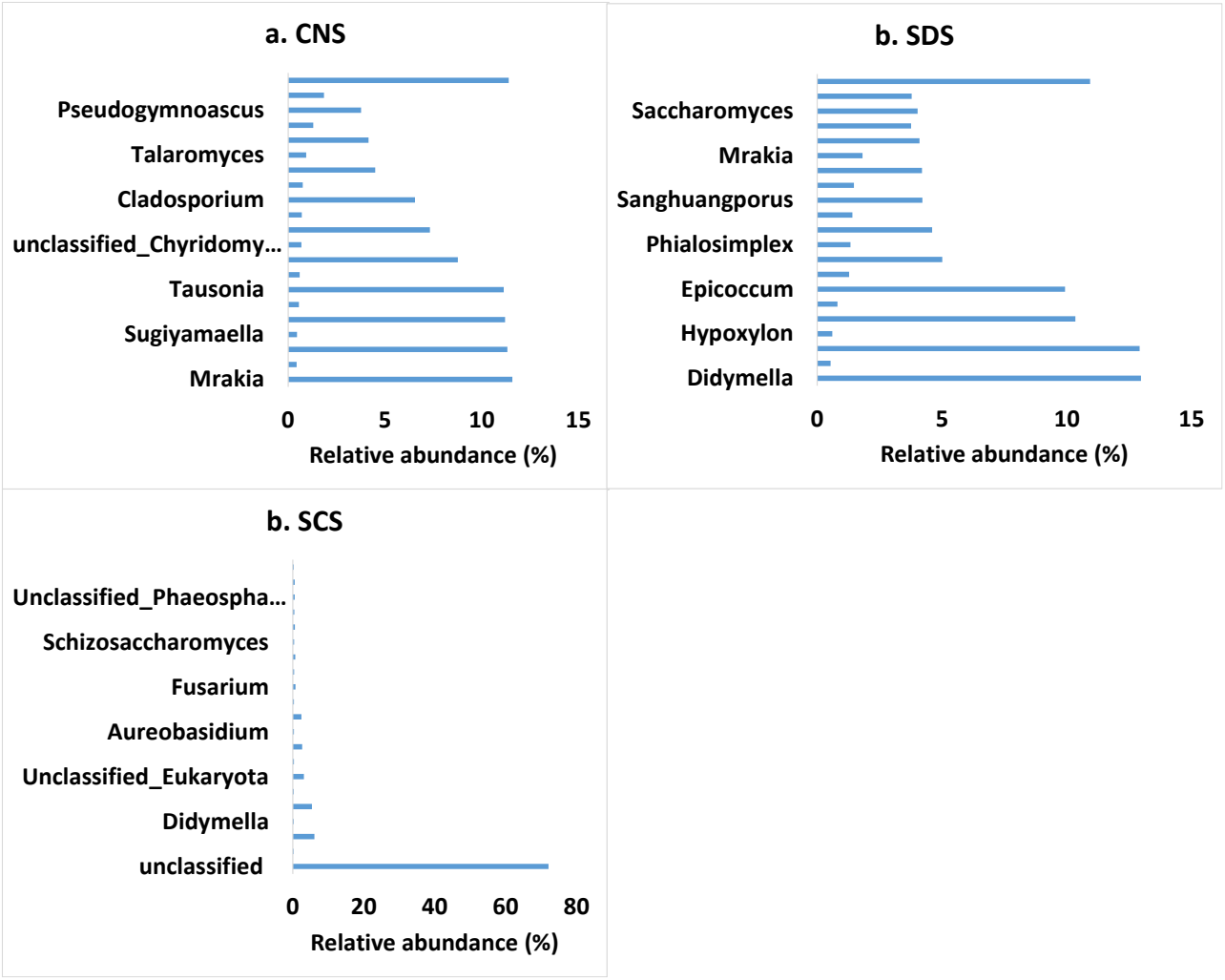
Supplementary Fig. 2 – Genus composition of (a) Clean Natural Soil, CNS (b). Short term Diesel Polluted Soil, SDS (c). Short term Crude oil Polluted Soil, SCS at F 0°C.



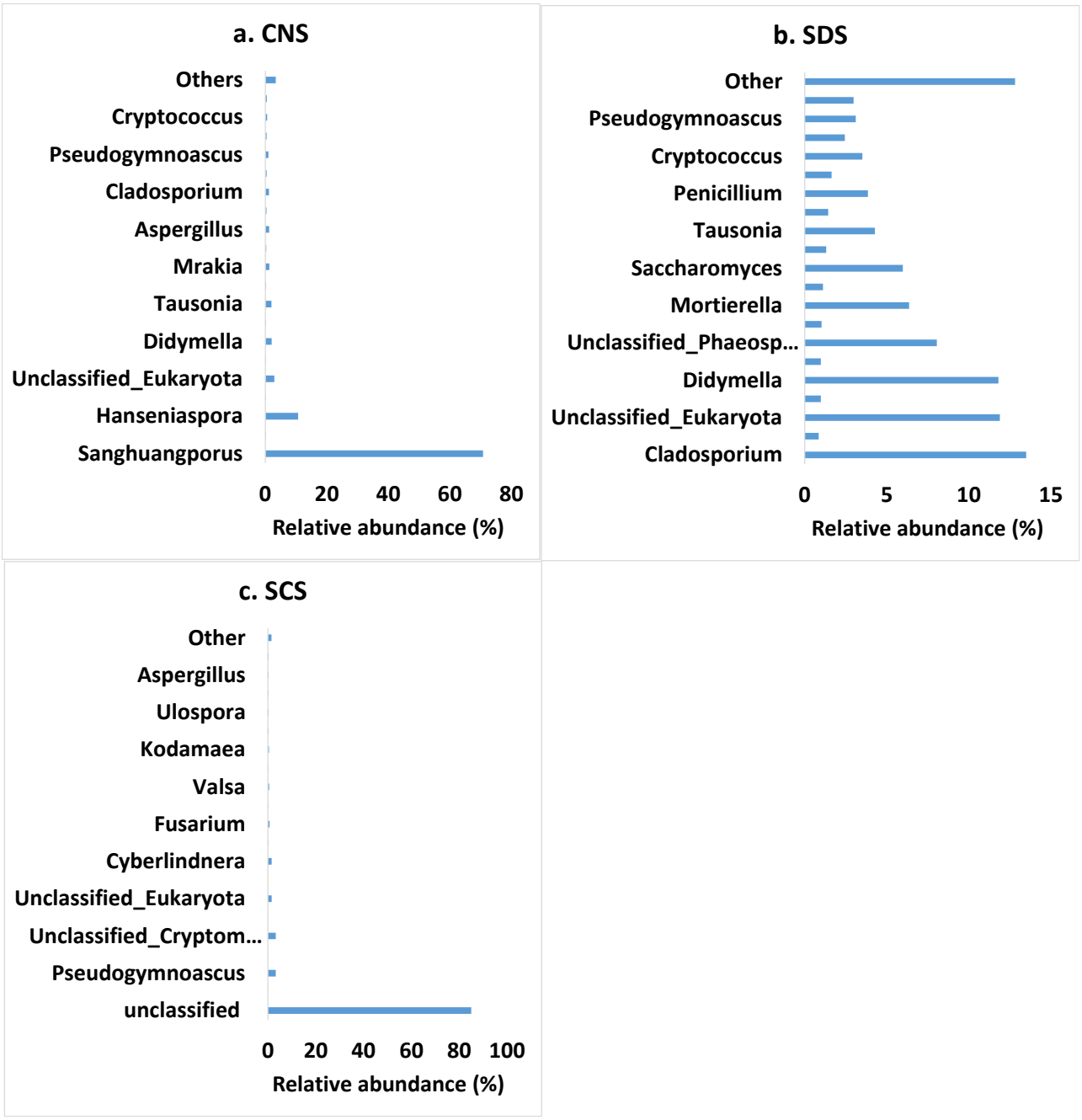
Supplementary Fig. 3 – Genus composition of (a) Clean Natural Soil, CNS (b). Short term Diesel Polluted Soil, SDS (c). Short term Crude oil Polluted Soil, SCS at F -10°C.



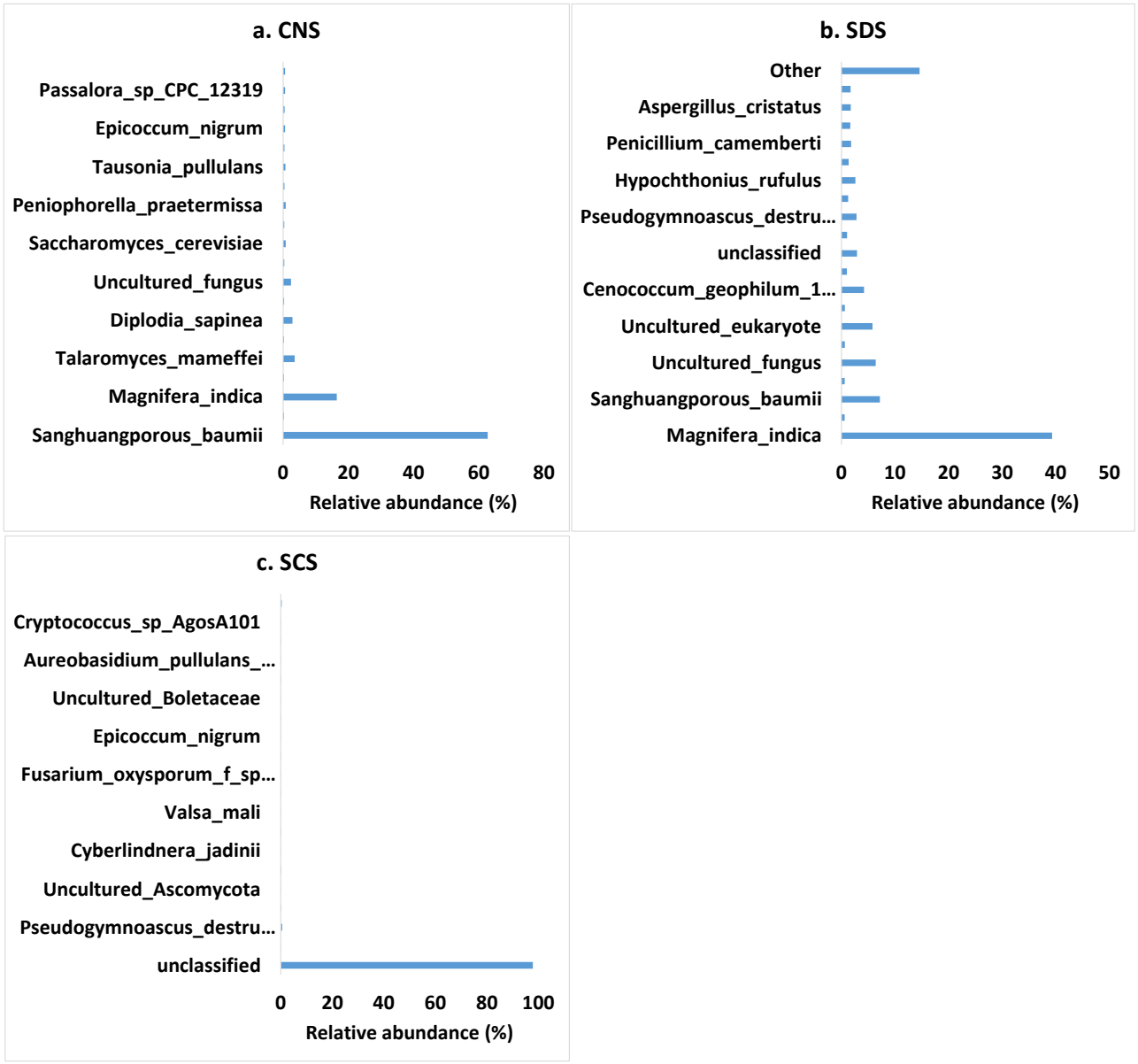
Supplementary Fig. 4 – Genus composition of (a) Clean Natural Soil, CNS (b). Short term Diesel Polluted Soil, SDS (c). Short term Crude oil Polluted Soil, SCS at F -20°C.



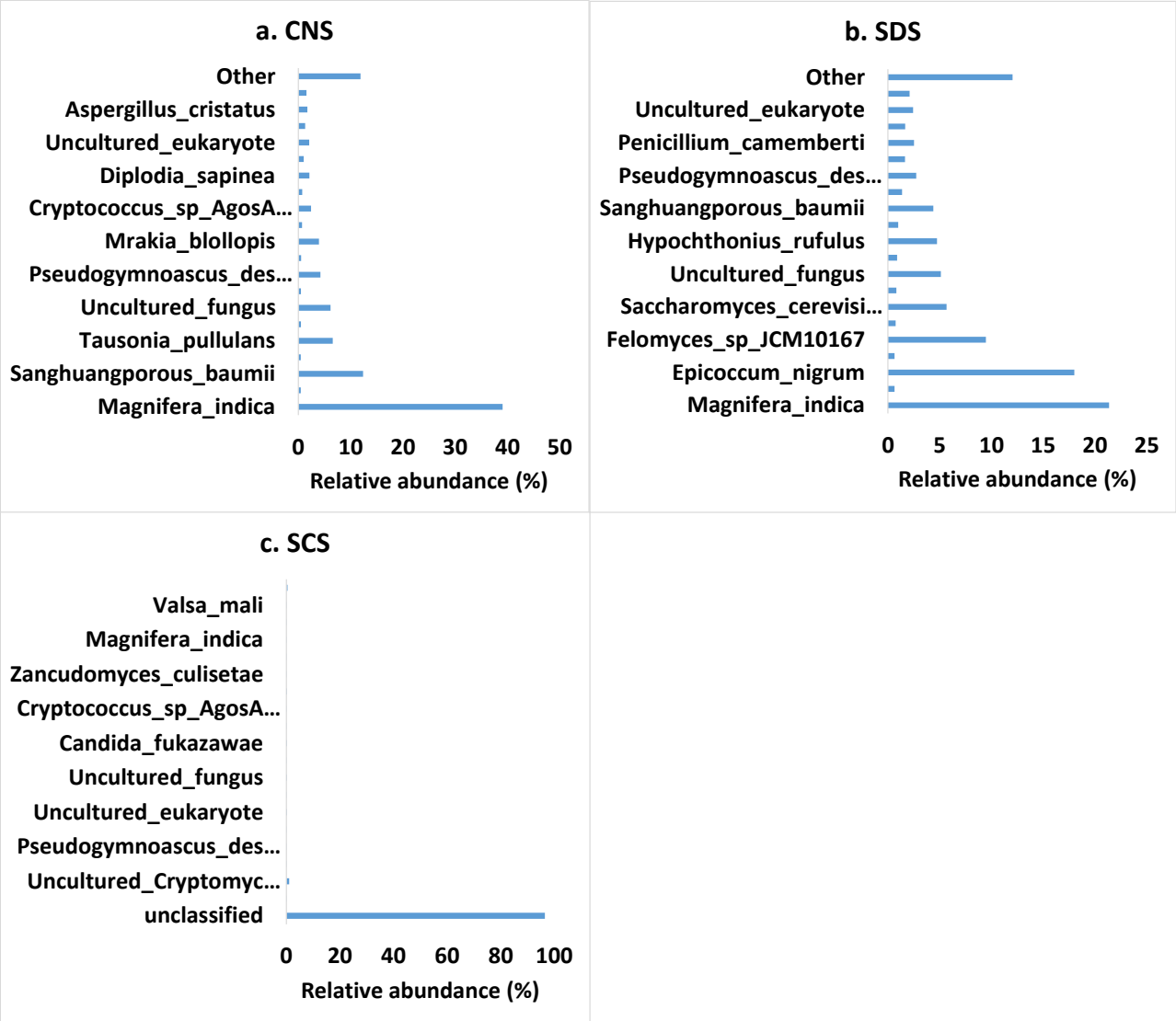
Supplementary Fig. 5 – Genus composition of (a) Clean Natural Soil, CNS (b). Short term Diesel Polluted Soil, SDS (c). Short term Crude oil Polluted Soil, SCS at T -10℃.



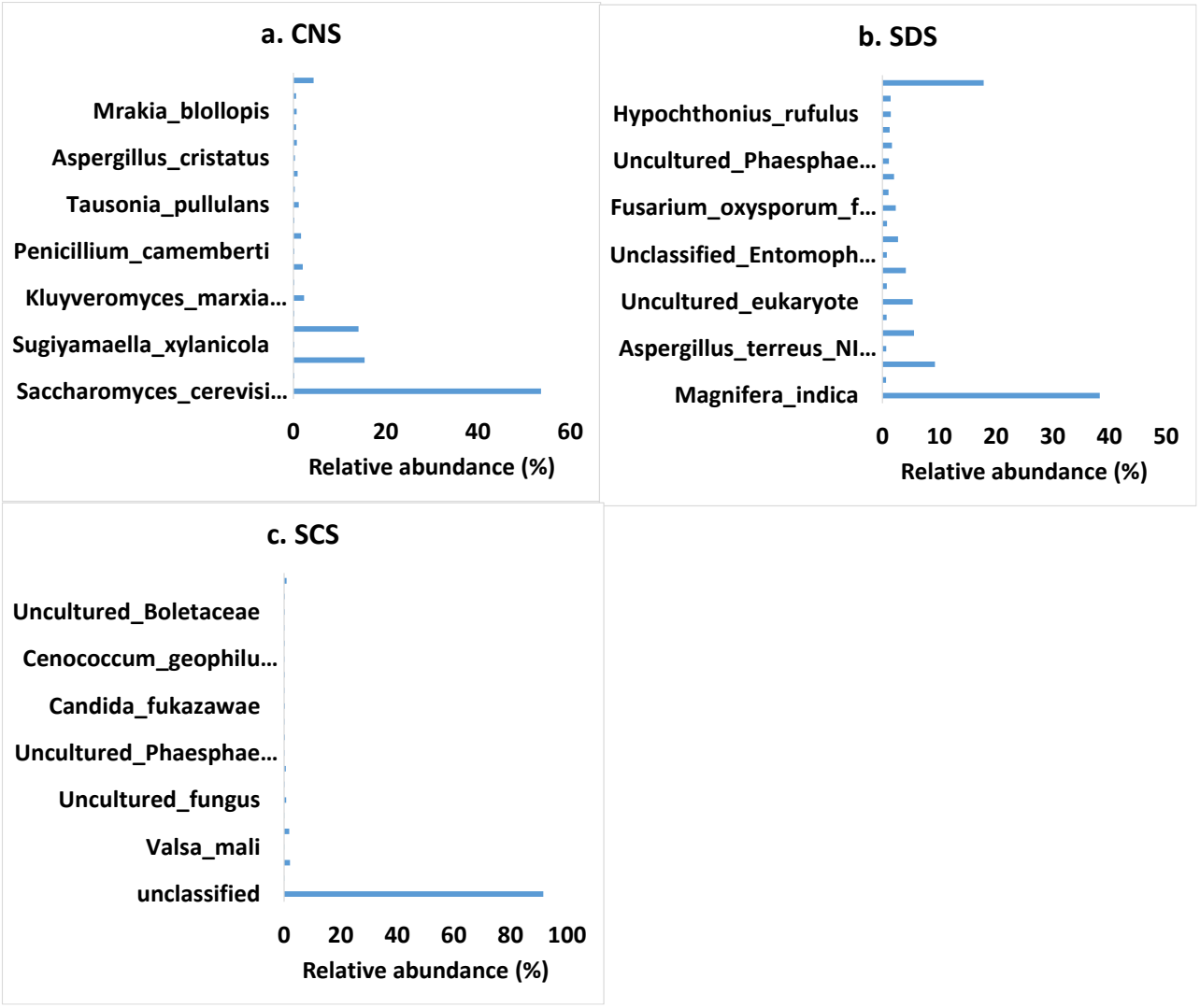
Supplementary Fig. 6 – Genus composition of (a) Clean Natural Soil, CNS (b). Short term Diesel Polluted Soil, SDS (c). Short term Crude oil Polluted Soil, SCS at T 0℃.



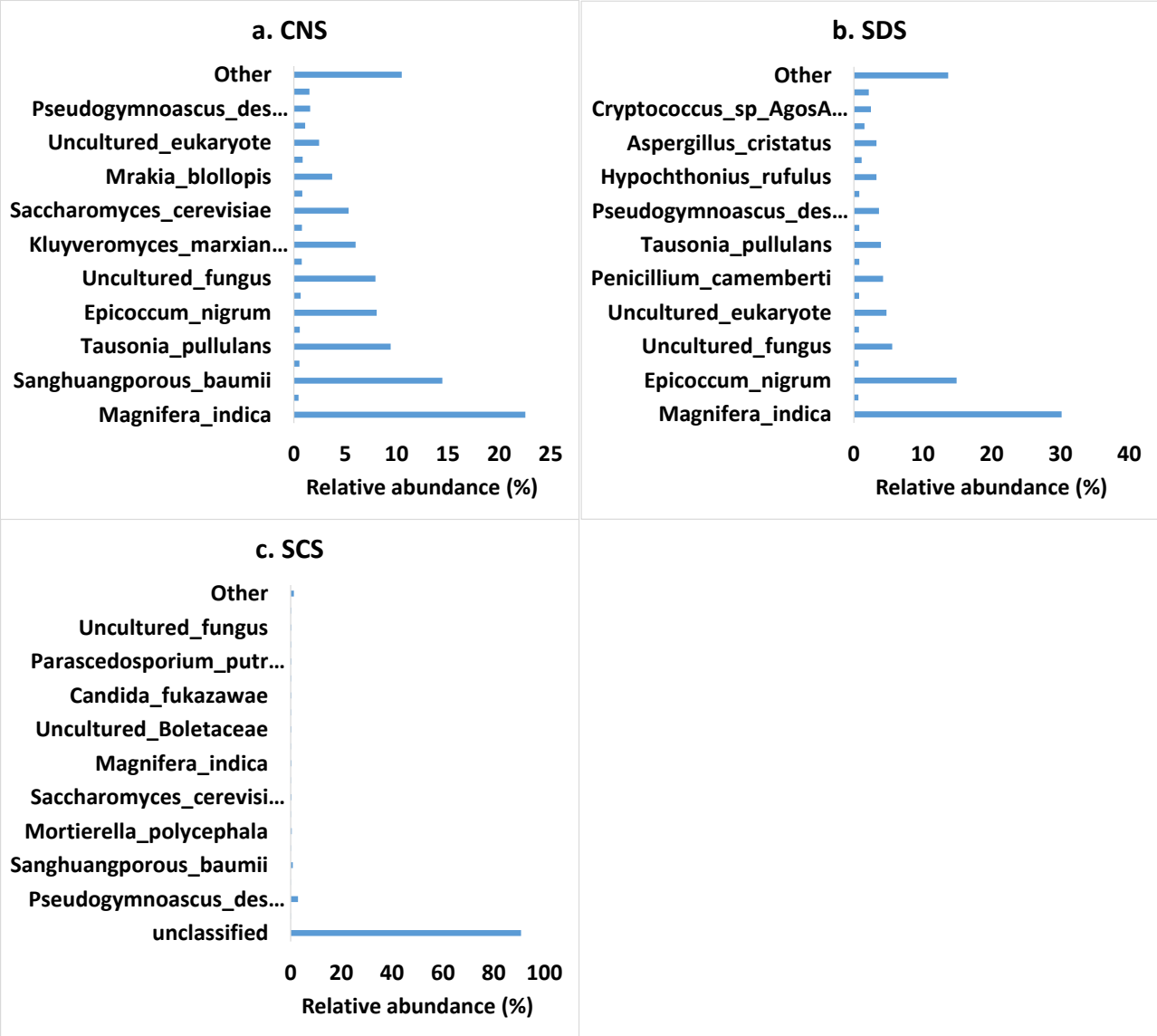
Supplementary Fig. 7 – Species composition of (a) Clean Natural Soil, CNS (b). Short term Diesel Polluted Soil, SDS (c). Short term Crude oil Polluted Soil, SCS at F 20℃.



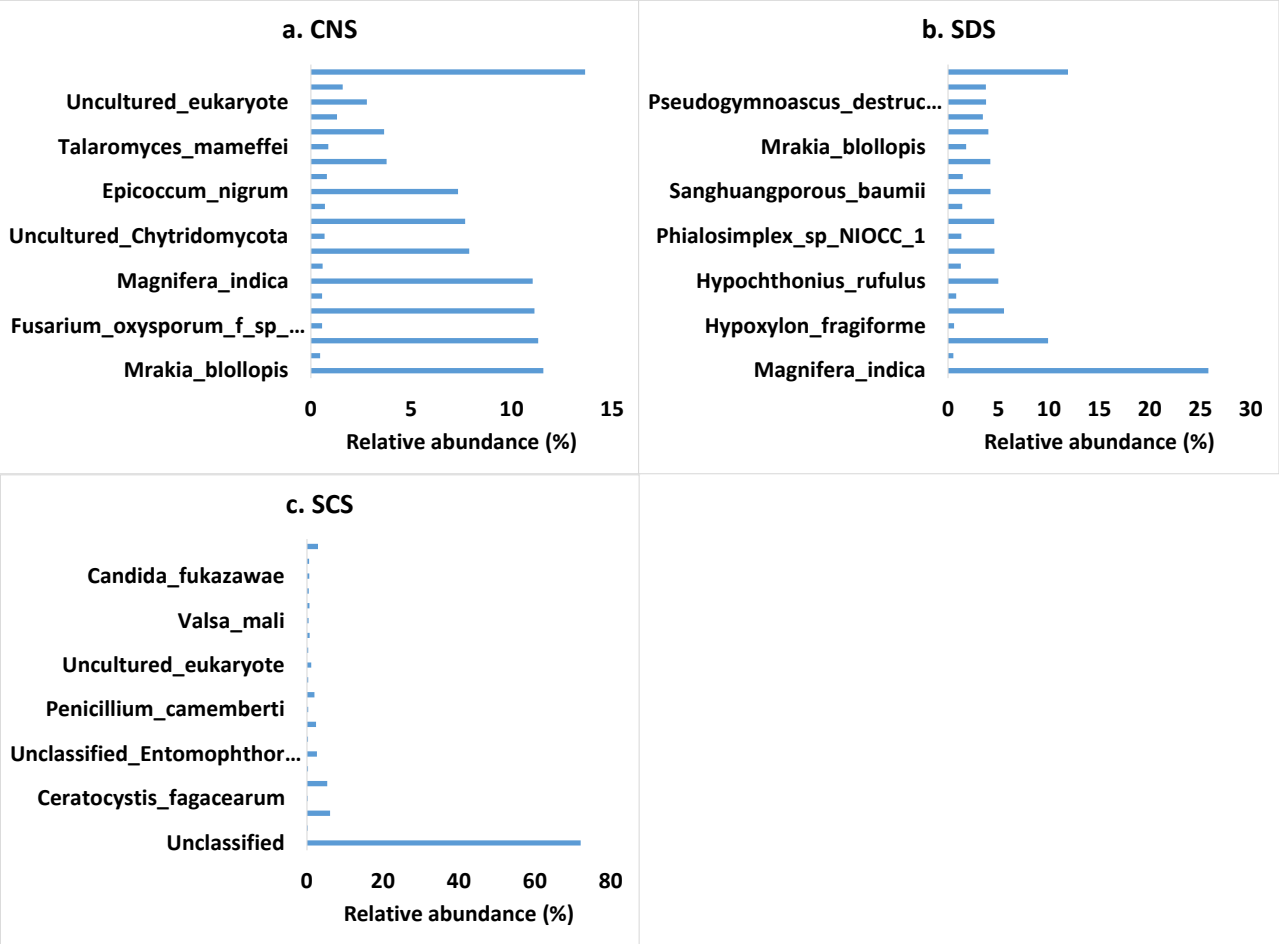
Supplementary Fig. 8 – Species composition of (a) Clean Natural Soil, CNS (b). Short term Diesel Polluted Soil, SDS (c). Short term Crude oil Polluted Soil, SCS at F 0°C.



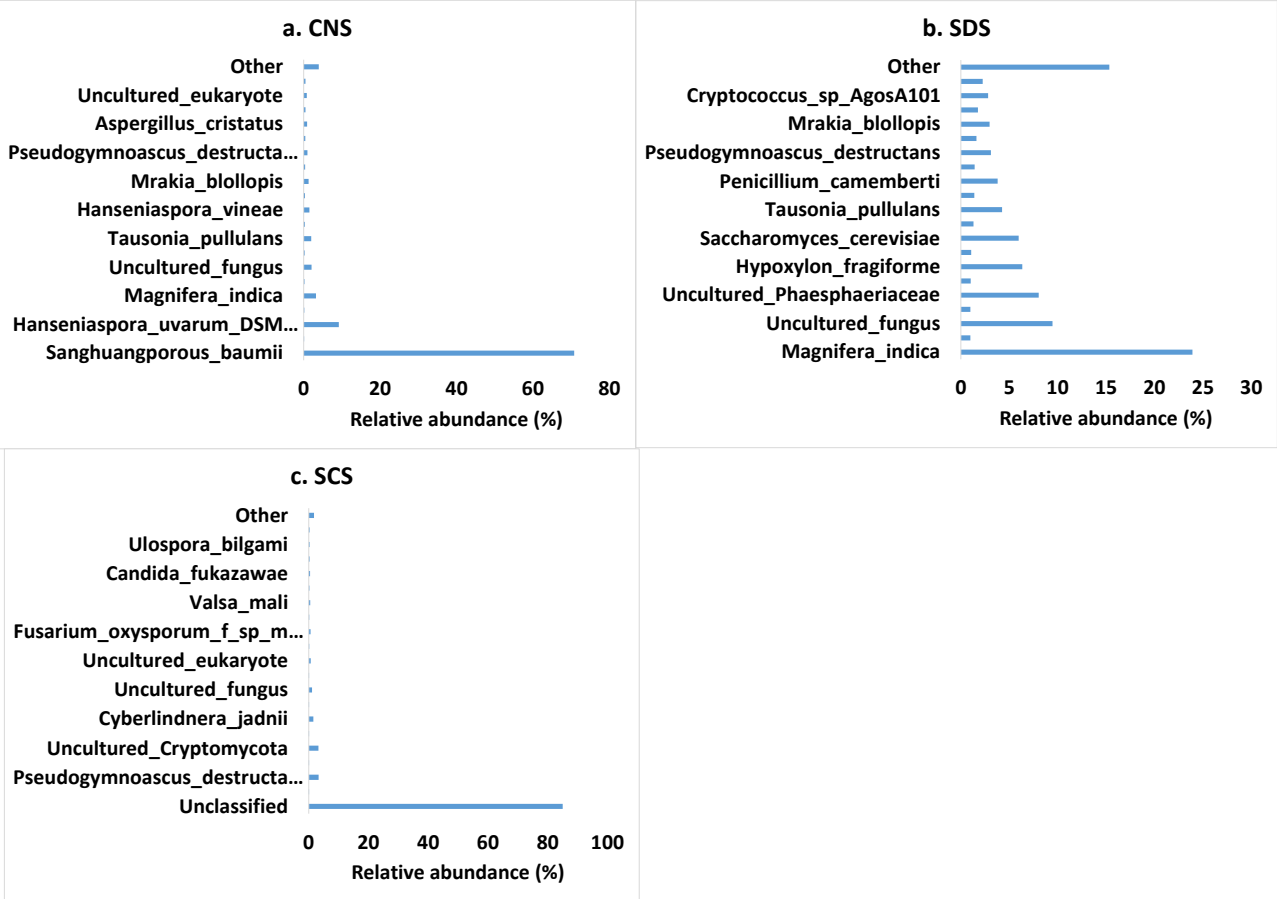
Supplementary Fig. 9 – Species composition of (a) Clean Natural Soil, CNS (b). Short term Diesel Polluted Soil, SDS (c). Short term Crude oil Polluted Soil, SCS at F -10°C.



Supplementary Fig. 10 – Species composition of (a) Clean Natural Soil, CNS (b). Short term Diesel Polluted Soil, SDS (c). Short term Crude oil Polluted Soil, SCS at F -20℃.



Supplementary Fig. 11 – Species composition of (a) Clean Natural Soil, CNS (b). Short term Diesel Polluted Soil, SDS (c). Short term Crude oil Polluted Soil, SCS at T -10℃.



Supplementary Fig. 12 – Species composition of (a) Clean Natural Soil, CNS (b). Short term Diesel Polluted Soil, SDS (c). Short term Crude oil Polluted Soil, SCS at T 0℃.