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Do the Culturable Microbial Groups Present in Cutaway Bogs Change According to Temporal Variation? Pilot Study Based on the Midlands in the Republic of Ireland

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Article

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Abstract: Cutaway peatlands in the midlands of the Republic of Ireland are rarely the focus of scientific studies. Due to peat extraction, the soil quality and related microenvironment is severely impacted. Returning them to a 'near natural state' would require greater insights into this ecological niche. The current work took the initiative to study microbiology of vast cutaway sites in the midlands of Ireland. Peat was collected over two seasons in January, February and April. Homogenised peat was aseptically cultured on a range of specific and non-specific culture media. Microbial enumeration, Gram staining and other microscopic observation of morphologically distinct microorganisms were performed. Total viable bacterial and fungal numbers were highest in February (1.33×10^5 CFU ml^{-1} and 5.93×10^6 CFU ml^{-1} respectively) and were lowest in April (1.14×10^3 CFU ml^{-1} and 5.57×10^6 CFU ml^{-1}). *Penicillium* spp. and *Trichoderma* spp. were common in all the sites. The highest values of phosphate solubilising index were recorded in peat collected in April (SI =3.167 & 3.000). Overall, there is a statistically significant difference ($p \leq 0.0001$) among the microbial numbers across the three months. This variation could be due to the temperature and pH difference in peat soil.

Keywords: Cutaway peatlands; aerobes; fungi; anaerobes; actinomycetes; restoration

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1. Introduction

Peatlands are water-logged ecosystems that can store thousands of years of carbon in the depths of their soil [1]. In order to gain land, modern methods strip the surface vegetation away and dry out the surface layers using large machines [1]. During the peat extraction process, original surface peat forming vegetation is disrupted. It eventually leads to peat drainage [2]. Drainage-based practices in agriculture and forestry has caused approximately 15% of peatlands to degrade worldwide [3]. The long-term effects of peat harvesting include peat compaction, formation of different vegetation types, manipulation of soil chemistry and erosion [1,2]. Furthermore, hydrological functions are impaired. It drops the water table level [4]. The combination of low water table level and high oxygen content can facilitate the accelerated rates of microbial decomposition and subsequently the CO_2 gas emissions increase [4,5]. When there is no longer an economic supply of peat at a particular site, it becomes rich in atmospheric carbon [2,6]. It leads to continuous greenhouse gas emissions [6].

Considering the major impacts of peatland drainage, the Republic of Ireland has launched several restoration projects across the country. One primary example is the

'Rehabilitation of cutaway peatlands in the midlands of Ireland'. It's objective was to recreate wetland nature in previously used industrial cutaway bogs [2]. The BOGFOR research program (1998-2005) was another Irish project. BOGFOR addressed the key components in afforesting industrial cutaway lands. The main challenges were soil heterogeneity and avoiding the dominance of competitor plant species [7]. Introducing peat forming plant species into the land has received the most attention in peatland restoration practices [8]. For restoration, plant community studies have been used widely while there is a paucity of studies applying soil microbiology of cutaway peatland ecosystems. Microorganisms can regulate many interactions with plants and the soil [9]. Proper understanding of the 'terrestrial carbon cycle' is required to implement novel restoration practices. Because soil microbial community greatly impacts the carbon cycle. Microorganisms mediate major steps of the terrestrial carbon cycle [10]. But there are no studies reported on different types of microbial groups in relation to numbers and temporal variation in cutaway bogs. Information about peat soil in the scope of microbiology have the potential to unravel unseen dynamics of the peat environment. Novel findings can introduce effective re-wetting schemes. Urbanová and Bárta assumed that methanogenic microorganisms in peat can act as an indicator to observe the environmental conditions [11]. The effect of re-wetting schemes can be monitored by the numerous, yet reliable information obtained by the methanogenic Archaea in peat. In conclusion, the study emphasized the importance of investigating the anaerobic microbial populations [11]. One recent study reported the bioindicator values of 'mites and vegetation' to assess the quality of peatlands. They observed significant changes in the number of species of the vegetation in recovering peatlands [12]. Likewise, the microbial numbers and communities in a drained peatland could be different than its pristine conditions (Pristine condition of a peatland is its original state, often refers to as 'near natural' condition as reference). However, not only methanogens but other groups of microorganisms are also pivotal in understanding the necessary steps of reclamation since they mediate certain reactions in the terrestrial carbon cycle. Such data can be employed as bio-indicators to monitor the restoration success of drained cutaway sites. It is necessary to monitor activities of a peatland ecosystem against baseline or reference data to assess the restoration progress. It is something inadequate in Western Europe [13]. Throughout restoration, the drained lands reach 'near-pristine conditions'. Culture isolation is one way to find indicator organisms. While this is a long-term goal, our pilot study took the initiative to collect the necessary baseline data of culturable populations to reach that goal in the future. If studies are focused on and microbial groups in the sense of modifying them as microbial/environmental indicators, the complexity of the restoration process can be gradually minimised. Considering this 'potential' of microbes, our study presents the following aims and objectives for this pilot-research i.e.,(i) to collect baseline data of culturable microbial groups present in each cutaway site (ii) to compare the microbial numbers according to different groups in each site (iii) to demonstrate the possibility of microbial communities can act as an environmental measure for peatland restoration. The soil - microbial ecosystem must be well understood in the restoration process. Because it will not be achieved in a single day.

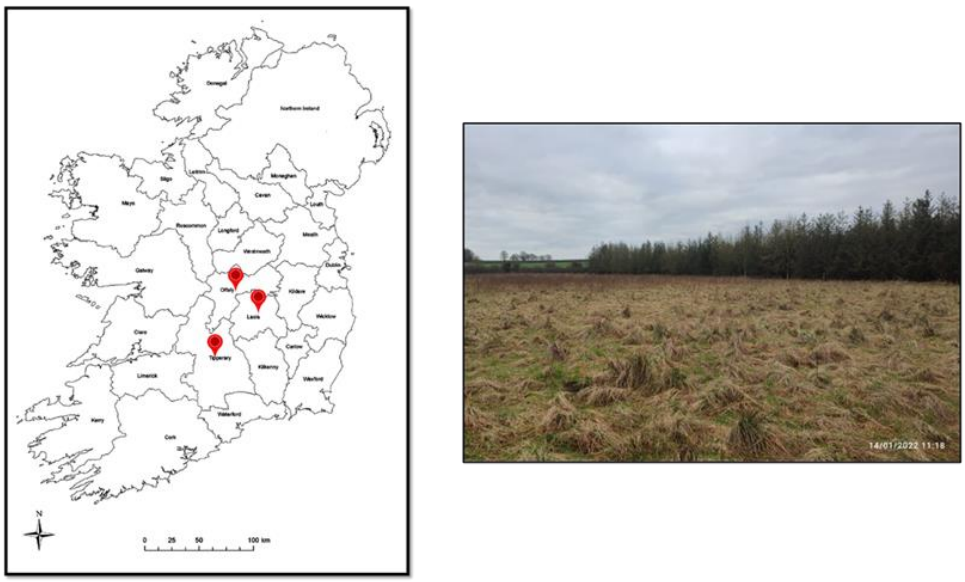
It is the first time in Ireland to conduct this type of a pilot microbiological study based on cutaway peatlands in the midlands (midland accounts for the main production in cutaway peatlands) [7]. The current work collected preliminary data of six different microbial groups according to temporal variation. The actual total microbial numbers might be higher than the ones illustrated (through culture isolation) in this paper.

2. Materials and Methods

2.1. Experimental set up and study sites

Samples were collected from study sites in County Offaly (53°9′46″ N 7°39′10″ W), County Laois (53°9′49″ N 7°37′18″ W) and County Tipperary located in the midland of Republic of Ireland (Figure 1). Peat across three cut- away-rough-grazing sites was

collected over winter and spring seasons (January, February and April) in the year 2022. The purpose of this experiment setting was to compare peat microbiota across the cutaway peatland sites and interpret if there are significant differences in the tested microbial parameters.



(a) (b)

Figure 1. (**a**) The map of Republic of Ireland denoting the sampling locations in the midlands; (**b**) The geographical view of the cutaway peatland located in County Offaly on January,2022. (The average temperature was 6°C).

2.2. Sampling

The vegetation type of the peat was carefully observed before the sampling process. Some of the observed vegetation types were *Sphagnum* mosses and sedges of which the lands were dominated with. Each site was divided into 10 strips having the form of zigzag. Ten composite soil samples were collected from each site to represent the whole site. Replicating the sample size would result in minimum errors. The depth of the soil was in the range of 0-15 cm. A standard soil knife was used to dig the soil into relevant depths. Peat soil 250-300g was collected to previously sterilized airtight containers. The samples were kept in around 4°C - 6°C.

2.3. Isolation of different types of culturable microorganisms

2.3.1. Isolation and enumeration of the total viable bacteria and aerobic bacteria

The soil samples were sieved through a 2 mm sieve to remove small stones, fauna and plant debris. Fresh peat (10.0 g) was suspended in 90.0 ml of sterile maximum recovery diluent. It was dispersed in a homogeniser at 140 rpm for 1 hour. The resulting suspension was diluted by serial, 10-fold stages. Pour plate technique (1ml aliquots) was performed for the dilutions 10^{-1} – 10^{-7} on Nutrient agar for the detection of total viable bacteria. The pH of the medium was 7.0 ± 0.2. Likewise, the serial dilutions were spread plated (0.1ml aliquots) on Nutrient agar and Tryptic soy agar (TSA) for the detection of aerobic bacteria. The plates were incubated in inverted position at 37 ±1°C. Colonies were counted after 48-72h incubation period. This procedure was repeated to peat soil from the three cutaway sites separately.

2.3.2. Isolation and enumeration of the culturable fungal population

Serial dilutions (10^{-1} – 10^{-7}) as described in section 2.3.1. were plated on half- strength Czapek-Dox agar medium. The agar medium was treated with streptomycin to inhibit the

bacterial growth. The plates were incubated at 25 ±1°C. The colonies were counted after 2-3 weeks of incubation period. Fungal colonies with different morphologies were sub-cultured on new Czapek- Dox agar media. The fungal spores, conidia, hyphae, sporangia were observed under light microscope. Primary identification of fungi was performed according to the descriptions and mycological keys in Gams and Bissett (1998) and Text book of Fungi [14,15]. This procedure was repeated to peat soil from the three cutaway sites separately.

2.3.3. Isolation and enumeration of anaerobic bacteria

The serial dilutions (10^{-1} – 10^{-7}) as described in section 2.3.1. were pour plated on anaerobic agar. The plates were incubated inside the anaerobic jars with the Microbiology Anaerocult® A (Reagent for the generation of an anaerobic medium) in it. The colony counts were recorded after 2-3 weeks of incubation at 25 ±1°C. Morphologically different colonies were subcultured three consecutive times to obtain pure isolates. Gram staining was performed for the purified isolates. This procedure was repeated to peat soil from the three cutaway sites separately.

2.3.4. Isolation of phosphate solubilising bacteria (PSB)

Sterilised Pikovskaya medium was initially prepared without dextrose (glucose) to avoid the sugar caramelisation. Filter sterilised dextrose solution was added to the sterilised medium separately. The dilution series (10^{-1} – 10^{-7}) as described in section 2.3.1. were spread plated on Pikovskaya agar. Control plates were set up by spreading 0.1ml of sterile maximum recovery diluent on Pikovskaya agar. The plates were incubated at 30±1°C for three weeks. Colonies with halozones were detected. The diameters were measured using a standard ruler. The reading error for a standard ruler with mm increments was +/- 0.1mm under optimal conditions. The uncertainity value was indicated for the diameter lengths in centimeters [16]. Phosphate solubilising index (PSI) for each colony was calculated using the following equation.

Phosphate solubilising index (PSI) = {(Diameter of the halozone + colony) cm/ (Diameter of the colony) cm}

2.3.5. Isolation of actinomycetes

Serial dilutions (10^{-1} – 10^{-7}) as described in section 2.3.1. were plated on starch casein agar. The plates were incubated at 25 ±1°C. Colonies with different morphologies were sub cultured after 2-3 weeks of incubation. All the bacterial colonies with different morphologies were subjected to subculture by three successive streak isolations to obtain pure cultures. Parallelly, Gram-negative and Gram-positive bacteria were differentiated by Gram's staining.

2.3.6. Glycerol stock preparation

40% (*v/v*) glycerol stock solution (200ml) was prepared by mixing glycerol (80ml) with de-ionized water (120ml). The stock solution was autoclaved. Pure isolates were obtained from total viable bacteria, aerobic bacteria, fungi, anaerobes, phosphate solubilizing bacteria and actinomycetes. Each pure bacterial isolate (single colony) was inoculated in Nutrient broth overnight. Fungal colonies were inoculated in Czapek-Dox modified broth. Overnight pure culture (500µl) was banked in sterilized 40% (*v/v*) glycerol solution (500µl) in sterile cryovials. They were gently mixed and labelled. Cryovials were stored at -80°C.

2.4. Measuring the soil pH

The pH values of each peat sample were measured using the soil survey standard test method. 1:5 soil: water suspension (*w:v*) was prepared as followed. 10.0g of peat was weighed into a clean duran bottle. 50 ml of de-ionized water was added into it. The soil suspension was mechanically shaken for 1 hour at 15rpm (LABWIT, ZWYR-D2402, Shanghai, China). The pH meter was calibrated prior to obtaining the readings. The electrode was immersed into the soil suspension and pH values were recorded.

2.5. Statistical analysis

Prism version 9.4.0 Graph pad Software, Inc. was used to produce the graphs and the statistical analysis. All the samples were analysed in triplicates. Data were presented in a logarithmic scale and error bars of all figures were presented using the mean with standard deviation (SD). Multiple comparison analysis was performed using Tukey's test unless otherwise stated. Statistical significance differences of the microbial population numbers were analysed using one -way analysis of variance (ANOVA) and two-way ANOVA with Tukey's post hoc-test.

3. Results

3.1. Total viable bacterial (TVB) and the aerobic bacterial (AB) population

The microbial numbers illustrated in this paper were based on a certain 'land use' type in peatlands. All the three sites described in this section were categorised under cutaway, rough and grazing. These three sites were located in different venues in the midlands. But their vegetation and the 'land use' types are quite similar. Therefore, our study made an attempt to compare the different types of microbial groups in three different climatic changes and how they vary with each group of microorganisms. The Average pH of peat soil in January, February and April was 4.15 ± 0.03, 6.03 ± 0.05 and 5.35 ± 0.04, respectively.

According to Figure 2a, greater number of total viable bacteria (1.33×10^5 CFU ml^{-1}) was recorded from the cutaway site which the samples were collected in February. There was a statistical significance difference ($p \leq 0.0001$) between the numbers of total viable bacteria across the three sites on January, February and April (Figure 2a). The total bacterial number was 1.14×10^3 CFU ml^{-1} in April. It was the lowest quantity among the three time points. This pattern was not observed for the aerobic bacteria. The numbers of aerobic bacteria were 1.71×10^5 CFU ml^{-1} and 1.20×10^5 CFU ml^{-1} in April and February, respectively (Figure 2b). However, there were slight differences in aerobic number across the three sites (Figure S1). According to Figure 2b, the comparison of TVB with AB populations indicates interesting findings. There was statistical difference between cutaway samples collected from January and April in terms of TVB and AB populations ($p \leq 0.0001$). Furthermore, there was a statistical difference between the February sample to the rest of the cutaway sites ($p \leq 0.05$). Peat collected in January and April showed higher AB population than the TVB. This phenomenon was opposite in February(Figure 2b). The ratio of AB to TVB was greater than 1.4 in January and April. It was less than 1 in February (Figure S2).

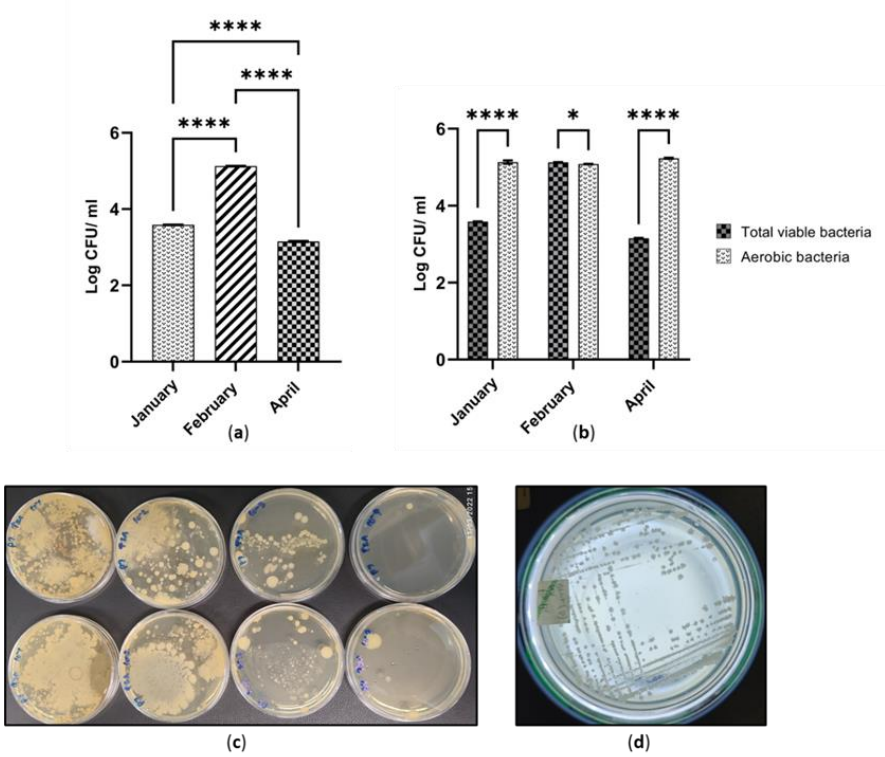


Figure 2. (**a**) Number of total viable bacteria across three cutaway sites cultured in three different time periods; (**b**) Comparison of total viable bacteria and aerobic bacteria, ns, not significant ($p > 0.05$); *$p \leq 0.05$; **$p \leq 0.01$***$p \leq 0.001$; ****$p \leq 0.0001$; (**c**) Initial serially diluted pour plate isolation of TVB in cutaway peat collected in February; (**d**) Typical viable bacterial pure culture isolated in January.

3.2. Morphology of bacteria under light microscopy

The microscopic observation of pure bacterial isolates revealed most were predominantly Gram-positive rods arranged as chains. (Figure 3a) They could be an indication of *Bacillus* spp. Cells with central endospores and sub-terminal endospores (Figure 3b) were detected. The proportion of Gram-negative (Figure 3c) isolates was comparatively less than that of Gram-positive bacteria. Occasional Gram-negative short rod (Figure 3c) cells were also detected.

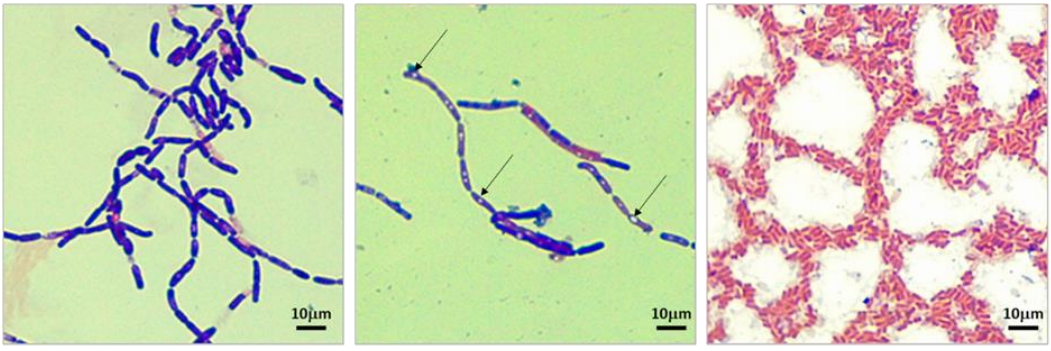


Figure 3. Light microscopy of subcultured bacterial isolates under oil immersion lens (10 x 100). (**a**) Gram-positive bacilli isolated in April cutaway site, (**b**) Gram-positive bacilli with sub-terminal endospores, (**c**) Gram-negative short rods isolated in April cutaway site.

3.3. Variation of fungal population in three temporal changes in different cutaway sites

Numerous types of morphologically different fungal species were isolated from all three sites. The sub-cultured fungal isolates were phenotypically different in colour, shape, size and the colony formation. According to Figure 4a, the fruiting body of *Penicillium* spp. was observed under the light microscope. It was detected in all the three cutaway sites. The growth rate of *Penicillium* spp. was comparatively higher than the other fungal species. Colonies of *Penicillium* spp. appeared on the Czapek- Dox agar during the first five days of incubation (Figure 4b & 4c). But most of the other fungal species required two weeks of incubation for the colony development.

The highest number of fungal population was found in February. April sample denotes the lowest amount of fungal population. Those values are statistically different ($p \leq 0.0001$). In accordance with Figure 4d, the fungal populations significantly differ with each other during the three time periods. There was also a statistical difference between January and February ($p \leq 0.01$). Confirming to Figure 4e, the fungi to viable bacteria (F / TVB) ratio was 1.05 and 1.14, in peat collected in January and April. The F/ TVB ratio in February was significantly lower than the rest. This value was less than 1 which was an indication of higher proportion of viable bacteria (0.76).

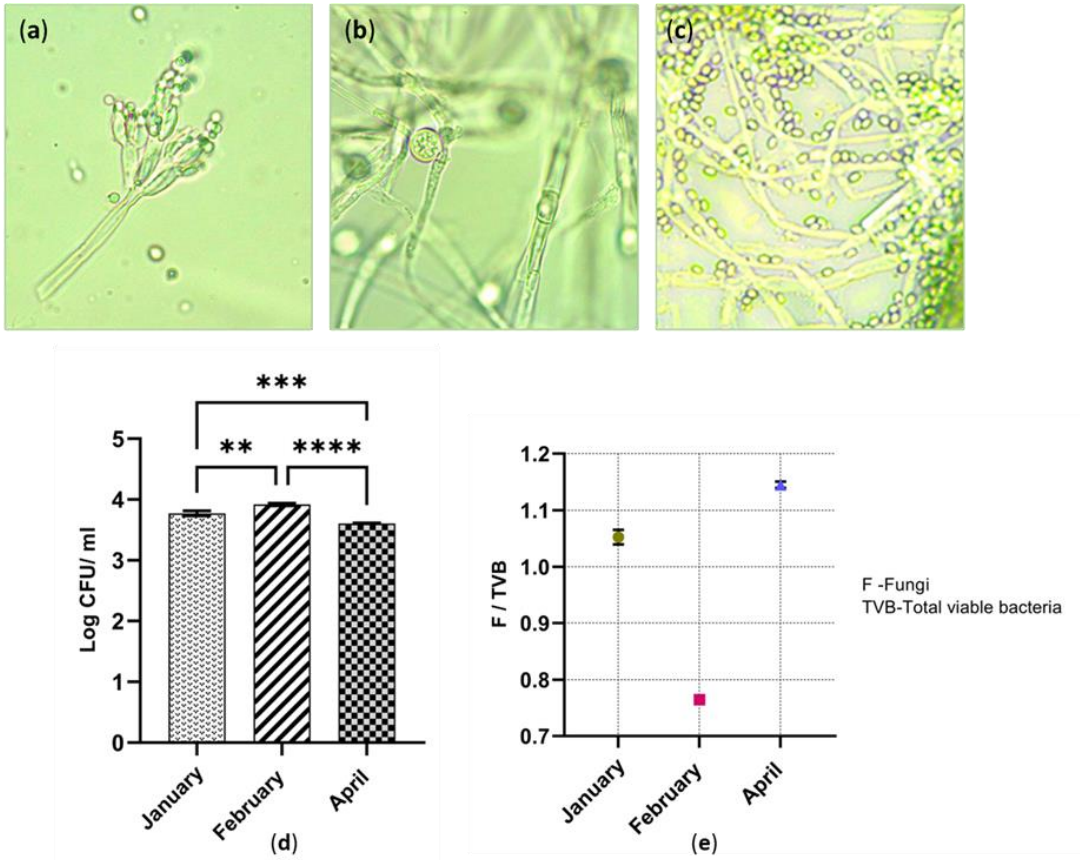


Figure 4. (**a**) Image of *Penicillium* spp. conidia- bearing structures under light microscope (10x100); (**b**) Image of spores inside sporangia under light microscope; (**c**) Image of *Trichoderma* spp. spores under light microscope (10x100); (**d**) Fungal population across the three cutaway sites, ns, not significant ($p > 0.05$); *$p \leq 0.05$; **$p \leq 0.01$***$p \leq 0.001$; ****$p \leq 0.0001$; (**e**) The ratio of fungi to viable bacteria in each cutaway site.

3.4. Variation and enumeration of anaerobic bacteria across the three sites

The maximum depth of peat collected was 15cm. Therefore, the following anaerobic microbial numbers represent the peat layer above 15cm of depth well. Considerable green colour colony development was achieved after 3 weeks of incubation. Gas formation was primarily detected at the bottom of the petri dish. Some of the colonies developed at the

bottom of the anaerobic agar layer. Other colonies were grown in the middle and on the surface of the agar medium. The abundance of anaerobic bacteria across the three study sites during January, February and April was illustrated in Figure 5a. Peat collected in January was the richest in anaerobic number (1.14 x 10^5 CFU ml^{-1}). This value was significantly higher than the rest ($p \leq 0.0001$). On the contrary, there was no significant difference in anaerobic bacterial numbers between February and April ($p > 0.05$). Those values were lower than the number in January by approximately one order of magnitude. The predominant organisms were Gram-negative short rods in all the samples. Occasional Gram-positive short rods were detected. Some isolates showed bizarre swellings in the middle of the bacterial cell. Some isolates exhibited terminal endospores.

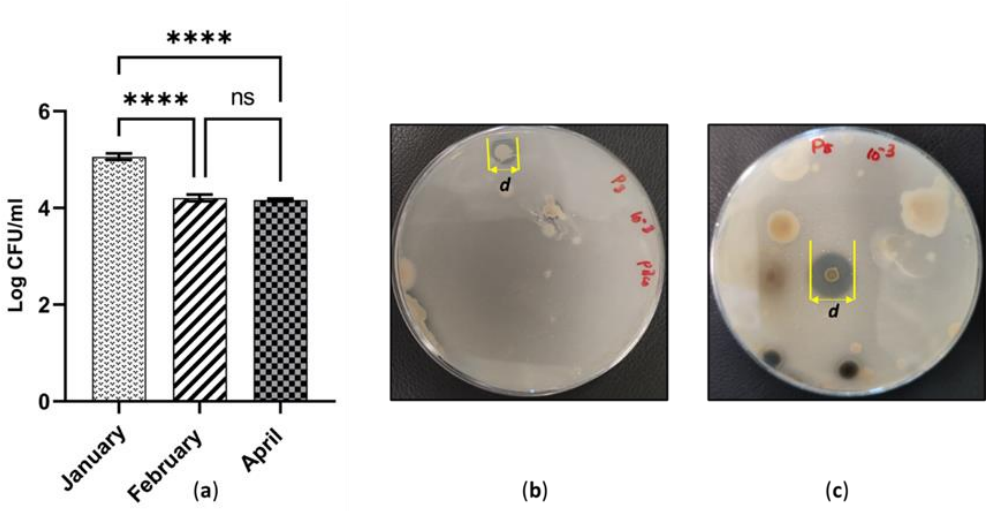


Figure 5. (**a**) Quantity of anaerobic population across cutaway sites in three different time periods, ns, not significant ($p > 0.05$); *$p \leq 0.05$; **$p \leq 0.01$***$p \leq 0.001$; ****$p \leq 0.0001$; (**b**) Halozone development due to phosphate solubilisation by PSB isolated in January; (**c**) Halozone development due to phosphate solubilisation by PSB isolated in April.

3.5. Phosphate solubilizing activity in three different time periods

Phosphate solubilising activity was determined using the Pikovskaya medium. It employs β-tri- calcium phosphate as the sole source of insoluble, inorganic phosphate. The concentration of the phosphate source and the pH of the medium were at a constant throughout the experiment. Therefore, phosphate solubilizing index was interpreted for the phosphate solubility at pH 7.0. Interestingly, phosphate solubilizing bacteria was detected in all the three sites. The majority of the PSB isolates took 2-3 weeks to reach the maximum diameter of the halozone. According to the Table 1. PSB which has a remarkable ability to solubilize calcium phosphate, were isolated in April (SI=3.167 & 3.000 respectively). Comparatively, the least ability to demonstrate phosphate solubilization was recorded in January (SI=1.25 & 1.26 respectively). It was noted that approximately 77.7 % of the PSB isolates demonstrates PSI values in the range of 1-2 and 28.57% above SI =2.

Table 1. Phosphate solubilizing index of PSB isolates after 3 weeks of incubation.

PSB Isolate	Average diameter of the colony(cm)	Average diameter of the colony + Halozone (cm)	Phosphate solubilization index (PSI)
P3S1	1.0 ± 0.1	1.25 ± 0.1	1.250
P3S2	0.95 ± 0.1	1.2 ± 0.1	1.263
P3S3	0.35 ± 0.1	0.5 ±0.1	1.429

P3S4	0.4 ± 0.1	0.525 ± 0.1	1.313
P7S1	1.05 ± 0.1	1.425 ± 0.1	1.357
P7S2	0.5 ± 0.1	0.65 ± 0.1	1.300
P8S1	0.5 ± 0.1	1.5 ± 0.1	3.000
P8S2	0.6 ± 0.1	1.9 ± 0.1	3.167
P8S3	1.25 ± 0.1	2.0 ± 0.1	1.6

[1] The uncertainity value (±) was indicated with the average diameter measurements.

3.6. Detection and isolation of actinomycetes

Colony development on starch casein agar was achieved after 2-3 weeks of incubation. Colony development was comparatively slower than the typical bacteria. Many colonies exhibited the appearance of halozones. The halozones remained colourless when the plates were treated with iodine solution. The typical characteristic colonies were 'pellet' like and pigmented (Grey, pink, maroon, yellow and orange). The presence of substrate mycelium and the aerial mycelium were prominent. Gram-positive clusters of branched filamentous bacteria were detected under light microscopy. They resembled filamentous fungi.

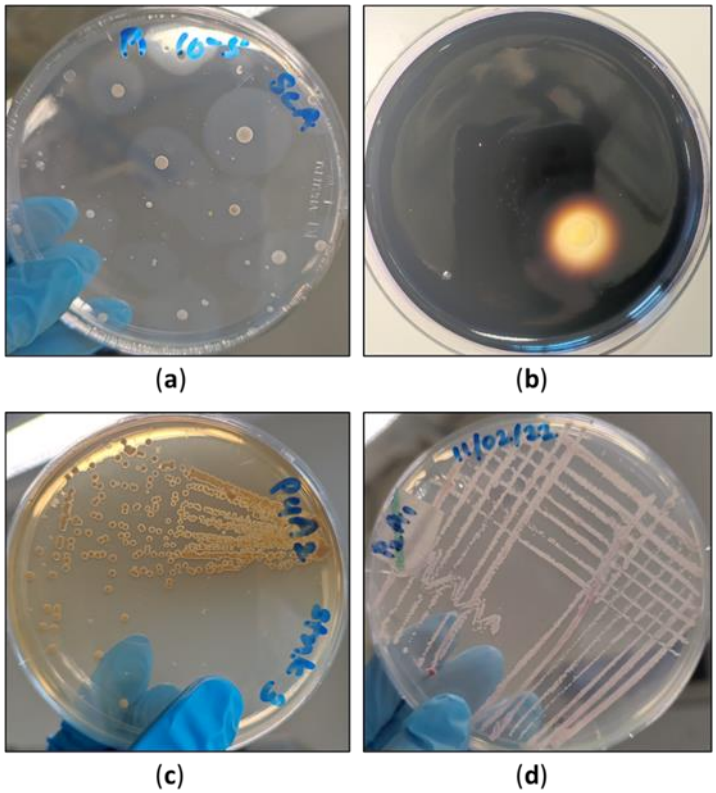


Figure 6. (**a**) Halozones developed due to starch hydrolysis by the bacteria grown on starch casein agar; (**b**) Visible clear zone around an actinomycete colony after the addition of iodine; (**c**) Pigmented actinomycete isolate obtained from the cutaway site in April; (**d**) Pigmented actinomycete isolate obtained from the cutaway site in January.

4. Discussion

Cutaway peatlands in general are former bogs [1]. They were converted to drained lands due to extensive use of land for peat extraction [2]. From soil structure to microbial environment is altered in cutaway peatlands due to its 'land use'. The differences of the vegetation that occur during the formation of cutaway land have been studied in several

attempts [1,17]. While it was proven to be an ideal approach to restore the degraded peatland like cutaway sites, finding a solution from a microbiological point of view can be promising in setting up effective re-wetting schemes. Therefore, through this study baseline data of different microbiological groups were collected and analysed in three cutaway sites in the midlands over 3 months (2 seasons). Vegetation pattern in these lands showed some similarities. Peat soil from each site was collected in January, February and April. The two physiological factors considered are temperature and pH of the peat soil. The average temperature in peatlands during the three months were 6°C, 7°C and 10°C respectively. For the culture isolation of this study, both selective and general-purpose media were used. The study strongly intended to differentiate the isolation procedure for culturable microbial populations in peat. However, the aim of this study was to give optimal culture conditions in a laboratory and adapt the natural peat microorganisms to grow as regular microorganisms. The isolation of unculturable populations would require some adjustments such as adjusting the pH of the medium, temperature, preparation of media using peat extracts and long incubation periods.

In February, both the total viable bacterial population (Figure 2a) and the fungal populations (Figure 4d) were greatest compared to the other two months. April records the lowest numbers of total viable bacteria (Figure 2a) and fungal population (Figure 4d). The temperature of these two climates were around 7°C and 10°C (in February and April respectively). While this temperature difference is apparent, the effect of temperature on the quantity of microbiota cannot be modelled without further data. However, the changing of total microbial numbers is not temperature dependent. Physiochemical properties of peatlands over time can provide added value to explain the differences of microbial quantities over time. Both total viable bacteria (Figure 2a) and fungi (Figure 3d) reflect a similar pattern in terms of microbial quantities. The numbers in three months are significantly different from each other. This could be due to the difference of temperature, pH and the nutrient availability of the soil. Peat collected in January and April are much more acidic (4.15 ± 0.03 and 5.35 ± 0.04 pH) than February (6.03± 0.05 pH). The pH of the culture media was 7.0 ± 0.2 which is a neutral value. Initially microorganisms were thriving in acidic soil for a longer time. They face a difficulty in adapting to a neutral environment. However, the pH difference (6.03-7.0pH) in peat and the culture media was lowest in February. The higher bacterial and fungal growth recorded in February could be due to this better chance of microbial adaptation to neutral environment (7.0pH). But the numbers of aerobic bacteria do not resemble the same pattern as the total viable bacteria. There is no significant difference between January's and February's aerobic population. Aerobes strongly depend on the availability of oxygen. During the sampling process if a higher proportion of peat soil was collected from the top layers, the possibility of isolating more aerobes is higher. In current study short incubation periods were employed to isolate aerobic and total bacteria. It was necessary to target the culturable organisms. A successful growth of aerobes was observed after 48 hours of incubation [17].

A study conducted by Rebekka et al., (2007) examined the effect of vegetational succession on the fungal community and structure [18]. The effect was strongest for the peat soil collected from the surface horizons [18]. Differences of the fungal communities have been proven by denaturing gradient gel electrophoresis-fingerprinting [19]. Very few studies have actually detected such changes. Apart from the prokaryotic communities, eukaryotic organisms have also been addressed in the context of cutaway peatlands. In Ireland, some of the cutaway peatlands in midlands have been converted to wetlands because of reflooding. Based on such sites, phytoplankton communities were assumed to be a great tool to monitor the chemical water quality [18]. Abundance of organisms such as dinoflagellates, blue-green algae have reflected the water quality in cutaway sites located in the midlands. These sites were rich in phosphorus and other minerals. Phytoplankton communities act as indicators to monitor the level of water quality in wetlands [20]. Prior to this study Higgins and co-worker have analysed zooplankton species present in artificial lakes. These lakes were created on Irish cutaway peatlands. The study also revealed

that establishment of phytoplankton and protozoans is rapid when the cutaway lands are flooded [21]. The same principle can be applicable to microbial community. One report shows the microbial abundance acts as an early indicator to observe the changes in soil quality. The particular study has utilised peat microbes to measure soil carbon and nitrogen in peatlands [22].

The scientific literature states that microbial availability is greatly governed by the factors such as temperature, moisture, oxygen availability and the substrate concentration. But root exudates of plant matter can influence the growth of some soil microbes [23]. In this study most of the Gram- negative bacteria isolates were detected among the anaerobic bacteria (Figure 3c). One role of the Gram-negative bacteria includes the fresh carbon turnover [24]. Gram-positive isolates (Figure 3a & 3b) were mostly detected among the total viable bacteria across all the three sites. They have the potential to utilise recalcitrant carbon sources in peat soil [24]. A study carried out in Finnish cutaway sites has analysed the microbial community structures using the phospholipid fatty analysis. They observed statistically significant differences among the microbial community structures in Finnish soils. Peat collected in the 10-15 cm layer comprised higher proportions of Gram-positive bacteria than the Gram-negative bacteria [23]. Similar to our study, this study has also illustrated the fungi/bacteria (F/B) ratio respective to different depths and sites [23]. The F/B ratios (Figure 4e) recorded on our study sites are comparatively higher, similar to those determined in previous studies [25]. The proportion of fungi is higher in drained peatlands than the bare peat [23]. This can be taken into consideration in the restoration process. The ratio of fungi: bacteria could be a fine indicator of effective restoration practices to bring the drained lands into pristine condition. Cultivation and drainage can increase the abundance of fungi. But peat extraction could drive this force to inhibit the growth of fungi [23]. However, when practices such as ecological succession takes place on land the fungal species can degrade the plant litter. Thereby the F/B ratio gradually increases [23]. As per the results obtained in current study, the F/B ratio in three months are significantly different from each other (Figure 4e). To reason out this, previous plant succession steps and duration of peat extraction must be considered along with the temperature. A study conducted in China reveals that fungal communities are more sensitive than bacteria when they respond to drainage. The study concludes that the contribution of fungi is more significant than bacteria to build up the overall microbial activity [26].

Another peatland study based in Spain hypothesised that microbial community structure can be governed by the temperature and moisture content in seasonal changes. As in many microbiological studies, PLFA profiling confirmed that the F/B ratio was very low. And changes in microbiota with the temperature were noticeable. The study did not detect any correlation between the peat botanical origin and the microbial community. However, factors like temperature and aeration of peat had proven the exerted influence on microbial community composition [27]. An important microbial indicator like F/B (Figure 4e) is interpreted in current study. It is considered as a vital proxy for carbon transformations in peatlands [27]. As a pilot study, microbial parameters like these would be undeniably important in collecting baseline data for the continuation of this project.

The majority of studies based on peatland microbiology address the dynamics of anaerobic microorganisms. The reason is most methanogenic bacteria are anaerobes. They act as the key reason for greenhouse gas emissions [17]. The type of vegetation is another vital element influential on the greenhouse gas emissions [28]. One underlying reason (in general) is that lack of vegetation in abandoned cutaway peatlands encourages peat oxidation. It increases CO_2 emission [17]. Hypothetically, there is no exact single class of bacteria capable of breaking down the complex polymers in peat. A wide range of microorganisms are involved in the anaerobic degradation process in peatlands. Some microbes can produce methane gas in the anaerobic peat layer. The produced methane gas is utilised by other microorganisms residing in the aerobic peat layer [28]. Therefore, there is a rising necessity of exploring the anaerobic microorganisms in peat ecosystem. Because it

could build a platform to take measures to mitigate peatland drainage. A recent study conducted by Urbanová and Bárta have shown the importance of studying the anaerobic community of drained peatlands. Based on the abundance of methanogenic community in pristine, re-wetted and drained sites, some vital conclusions have been drawn. Methanogenic abundance in drained site could reach up to approximately a pristine-like state after the re-wetting schemes were applied. It proves that microorganisms are such good indicators which could reflect the restoration success in peatlands [11]. Therefore, the current study collected baseline data of abundance of anaerobic bacteria (Figure 5a) in three cutaway sites. The cutaway site in January records the highest number of anaerobic bacteria. It is significantly higher ($p \leq 0.0001$) than in February and April. The initial growth of anaerobic bacteria leads to the release of CO_2 to the anaerobic medium. When the CO_2 dissolves, carbonic acid is produced. Carbonic acid can make the anaerobic medium slightly acidic, although it is slightly neutral initially. Considering the initial pH of the peat soil, anaerobes from the January sample could have better opportunity to grow in a slightly acidic medium than the rest. This could be one underlying reason for the higher numbers of anaerobes recorded in January. Comparatively low numbers of anaerobes were recorded in February and April. Without further information regarding any previous re-wetting steps, only narrow conclusions could be made from the initial enumerations of the anaerobic bacteria in our study. However, the comparative data of microbial quantities and the pure anaerobic isolates could be quite useful to draw valid conclusions, when this study is continued ahead of its pilot stage in the future. A study carried out in Finland has focused the methane-cycling microbial communities in Finnish peatlands. Based on the abundance of methanogens and methanotrophs, the authors conclude that it will at least take up to about 10 years to restore the forestry drained peatland. They predicted it using the microbial indicators. Microbial indicators were isolated from the aerobic surface and the anaerobic surface of peat [29]. Few countries which are rich in northern peatlands has taken this approach. But Republic of Ireland has not entirely stepped into the application of microbial indicators to analyse the vegetation succession or the restoration succession after rewetting of cutaway peatlands.

Apart from the main characteristic microbial groups, actinomycetes and phosphate solubilising organisms share a great responsibility in the decomposition process in peatlands. Knowledge concerning the role of these specialised microbial groups in Irish peatlands and other countries is limited. One such study carried out in North America studied the effect of vacuum extraction of peat using microbes as biological indicators [30]. The difference between a natural peatland and a vacuum extracted peatland was compared using microbial populations including the actinomycetes [30]. Bacterial and the fungal populations were lower in vacuum extracted lands than the pristine- state lands [30]. The numbers of actinomycete populations were always lower than the typical bacteria which is also consistent with our studies. However, it is not accurate to assume that all the colonies grown on starch casein agar belong to the group of actinomycetes. Even saccharolytic organisms can grow on starch casein agar. Therefore, interpreting the colony counts on starch casein agar as a whole of actinomycetes number would not be entirely accurate. The population could represent both the saccharolytic and actinomycete organisms. In this present study, starch casein agar medium was used to isolate actinomycetes. The sole source of carbon is starch. But some actinomycetes are unable to utilise starch. They would not have been detected at some point. However, actinomycetes which utilise starch can release a significant amount of glucose (and other simple sugars) molecules to the medium. So, there is a possibility that actinomycetes which cannot utilise starch but can rely on the glucose released to the medium. The initial aim of the current study was to detect the presence of actinomycetes in these three cutaway sites using a standard selective medium. Starch casein agar was frequently used in previous studies solely based on actinomycetes. The basis of selecting the isolation media was to isolate the preliminary yet culturable microbial population first. In our study, several distinct microbial groups have been primarily isolated in relation to their numbers. The authors took morphology into

consideration as an initial step for primary detection of actinomycetes. But of course, if this study is proceeded ahead its pilot stage, a detailed characterisation of actinomycetes using the biochemical tests can be performed. The collective data of the actinomycetes can then be presented in a separate manuscript. Commonly isolated actinomycete species in previous research work include *Micrococcus*, *Streptomyces* spp, and *Nocardia* [30]. Culture characteristics of the actinomycete colonies (Figure 6) isolated in each month are distinct. Further classification studies are yet to conduct to identify to the genus level. It should be emphasised that the majority of the soil actinobacteria are likely to thrive in aerobic conditions [31]. Therefore, in this study when the peat taken in the below layers may have restricted the growth of strictly aerobic actinomycetes. Another study based on Finnish peatlands investigated the variation of actinobacterial populations among bogs. The composition of actinobacteria is correlated with the water table level. In conclusion, the writers state when the water table level is altered, obvious changes will be detected in the actinobacterial community [31].

According to the results discussed here the objectives of our study were achieved. That includes (i) baseline microbial data on Irish peatlands, (ii) comparison of microbial numbers (six different groups) in sites temporally and (iii) possibility of adapting microbes as early indicators to monitor restoration progress. Owing to this importance of studying the microbial diversity in Irish peatlands, preliminary data was gathered to continue studies and initiate setting up promising re-wetting schemes in the future. The subsequent reclamation will have significant impact on the Irish peatlands. It will aid to minimise the greenhouse gas emissions. The microbial parameters mentioned here will be a valid initiative for the future peatland research to study Irish peatlands in depth. Preliminary indicators like F/B is a tool to monitor peatland restoration over time. However, some possible future directions for peatland microbiology are worth mentioning. The pure isolates obtained from the current work can be sequenced to identify possible indicators in each season. The isolation of unculturable populations can be performed in a follow-on study by setting up bio-reactors which exactly mimics a peat environment. Data of unculturable microbial populations can be compared with the culturable population numbers. It can be presented in the future. Moreover, since this pilot research does not anticipate being a conclusive study for peatland microbiology, sample size will be increased during future work. Combined isolation-based study and metagenomics-based studies will provide a better picture of the total microbiota. Microorganisms other than bacteria and fungi should be focused on the future since their role could be notable.

5. Conclusion

In accordance with the results obtained here, there is an overall statistically significant difference between the microbial numbers (TVB, Aerobes, fungi and anaerobes) across three cutaway sites. Among the three time points, total viable bacterial and fungal numbers were highest in February when it is the coldest. The numbers of total viable bacteria and fungi were lowest in April when it is comparatively warmer. There is a statistically significant difference among the microbial numbers in three months. The difference of temperature and pH might be the reason for this significant variation of microbial number. *Penicillium* spp. and *Trichoderma* spp. are common in all the sites. The highest phosphate solubilising index values are recorded from the cutaway peat collected in April (SI =3.167 & 3.000). In view of the importance of studying the microbial diversity in Irish peatlands, preliminary data is generated while fulfilling the aims and objectives. This project also gives valuable future directions which will pave the way to set up promising re-wetting schemes to reduce greenhouse gas emission.

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Supplementary Materials: Figure S1: Number of aerobic bacteria across three cutaway sites cultured in three different time periods, Figure S2: The ratio of aerobic bacteria to total viable bacteria in cutaway bogs in three time periods.

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