# The microbial community profile of a snottite-like biofilm in an abandoned mine drainage tunnel in the Derbyshire Peak District, UK

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**Abstract**

The formation of biofilms on subterranean surfaces is a topic of significant interest since the characterisation of the constituent microorganisms can provide key insights as to the surface biogeochemistry across the micro to nano scale. Stringy biofilms (commonly referred to as snottites) have been described mainly from low pH (0.0 - 1.5) environments. However, stringy biofilms have also been observed in several soughs (lead mine drainage levels) in the Derbyshire Peak District where the surface water is typically near neutral. A snottite-like biofilm that was collected from Yatestoop Sough was visualised microscopically and the microbial community structure determined through DNA extraction and sequencing of the 16S ribosomal RNA genes present. The community contained sulphur cycling organisms of the genera *Thiothrix* (16.2%) and *Thiobacillus sp* (8.7%). The presence of *Thiothrix* and *Thiobacillus* may suggest that reduced sulphur within percolating waters may be more influential in the formation of snottite like biofilms than the acidity of these leachates.

## Introduction

Mines in Britain are hosted in a wide range of lithologies, and have been used to extract various natural resources, including lead, coal, sulphur, copper and gold. In these subterranean environments, microorganisms generally grow in biofilms that can be found coating the exposed surfaces or hanging off the walls and ceilings. Biofilms are a complex matrix containing microbial cells, mucilaginous

secretions and extracellular materials which aid in the persistence of these organisms within their environment (Flemming et al. 2016; Konhauser 2001; Boston, 2006). The differing geochemistries found in these many subterranean environments, including mines, give rise to a host of visually diverse biofilms living on the various mineral surfaces found there. A number of studies across Europe (Borsodi et al. 2012; Edwards et al. 2006; Macalady, Jones, and Lyon 2007; Pfendler et al. 2018; Popović et al. 2015) have attempted to characterise these mine biofilms but there have been no comparable investigations in Britain.

Biofilms that hang subaerially and superficially resemble stalactites but are 'jelly-like' and pendulous have commonly been referred to as 'snottites' (Pain, 1998). Most descriptions of snottites refer to their presence in highly acidic environments (pH 0.0 – 1.5) (Hose, 2004; Macalady, Jones, and Lyon 2007; Jones et al. 2012; Krawczyk-Bärsch et al. 2011). From a geochemical perspective, the formation of these highly acidic leachates is the result of the oxidation of sulphide minerals or hydrogen sulphide gas from sulphur rich pools (Macalady et al. 2008; Jones, Schaperdoth, and Macalady 2016). This in turn leads to the generation of microcrystalline gypsum on the cave wall surface which acts as a surface from which the snottite biofilm can develop (Jones et al. 2012). There is also evidence to suggest that snottites can play an important role in the wider ecosystem of caves, such as the use of snottites by nematodes to prevent predation by mites (Borgonie et al. 2010).

The present study describes a snottite-like biofilm structure that was collected in Yatestoop Sough, Derbyshire, a tunnel that drains a former lead mine. Between February 2000 and April 2018 the Environment Agency collected 66 samples from the sough tail (the outlet) and these ranged from pH 7.4 to pH 8.0 with an arithmetic average of pH7.7. Given the paucity of information with regards to the microbial community profiles of biofilms from British mines or snottites from near neutral environments, the aim of the study was to describe the microbial community of the sampled snottite-like biofilm.

## Methods

### Site description

The entrance to Yatestoop Sough is located near Darley Bridge, Derbyshire, on private land (Figure 1). Construction of the sough commenced in 1751 and it was driven for about 2300m, initially through shale and then into limestone with the aim of dewatering mines in the Winster -Elton area (Oakman, 1979; Warriner, 2000). The first 60m upstream from the tail is arched with dressed gritstone blocks and may be cut and cover but the remainder is cut through the local rock. This is the Bowland Shale formation, of Carboniferous (Asbian to Yeadonian) age, and belonging to the Craven Group (Earp et al., 1961; Waters et al., 2007). The shale is organic-rich and fissile, with mm-scale bedding and minor sandstone beds. It was deposited in relatively deep hemipelagic conditions in a restricted basin setting. The sandstone blocks used for facing the tunnel are assumed to have been sourced from the overlying Millstone Grit formation. Following granting of permission by the landowners the site was visited on 5 December 2019. The flow at the tail was not measured at the time but based on previous measurements was in the range 130-180 L/s.

<Figure 2 here>

The sough was traversed for about 500m from the entrance to Wet Shaft (Figure 2) and over this distance the passage is 0.5 to 1.0m wide and 1-2m high and the water depth ranged from 0.3 to 1.0m. There are extensive solid precipitates and soft biofilms on the passage roof and walls extending upstream from the end of the section dressed with gritstone blocks to Wet Shaft (Figure 3). The snottite-like biofilm described in this study was retrieved from close to the base of Wet Shaft (Figures 2 & 4).

**Collection of material**

Biofilm material was retrieved from the surface of the rock using a spatula that had been sterilised using 6% hydrogen peroxide and 0.4% peracetic acid spray (Redditch medical, UK) and dried thoroughly with paper towel. The material was then transferred to a sterile 50 ml tube. On return to the laboratory the sample was stored at 4°C and was processed within 48 hours. A small sub sample of the material was removed using a sterilised scalpel on return to the laboratory for microscopic analysis. The structure

was visualised by wet mount using the 400X objective of an Olympus BX40 microscope and Delta InSight image capture software.

**Snottite-like biofilm pH analysis**

Snottite material (~0.2g) was mixed 1:5 with sterile deionised water as per B.S.I (2005). The mixture was then shaken on an orbital shaker at 120rpm for 60 minutes, and the pH of the solution measured using a calibrated pH probe.

**Microbial community analysis**

A portion of the biofilm material (~1g) was transferred to a sterile tube. DNA was extracted from the biofilm using a DNeasy PowerLyser PowerSoil kit (Qiagen, US). Presence of DNA was confirmed using gel electrophoresis in a 1% TAE-agarose gel and quantified using Qubit HS DNA assay kit (Thermofisher Scientific, US). DNA sequencing was carried out using Illumina HiSeq technology for the identification of Bacteria (16S rRNA gene). Sequencing was carried out by Chunlab (South Korea) through amplification of The V4 region of the 16S rRNA gene, amplified using primers 519F (5'-CAGCMGCCGCGGTAA-3') and 785R (5'-TACNVGGGTATCTAATCC-3') for bacteria and archaea. The retrieved sequences were then trimmed, paired and chimeras removed using the MOTHUR suite of packages (Schloss et al, 2009). Operational taxonomic units (OTUs) were then assigned by CD-HIT clustering at a 95% cut-off and taxonomic assignments made using the EzTaxon database (Chun et al, 2007). The raw sequences reads were submitted to NCBI Genbank under Project ID PRJNA 868918.

**Results & Discussion**

Sequencing of the 16S rRNA gene resulted in 79,996 valid reads which were further classified into 2,197 operational taxonomic units (OTUs). Of these OTUs, 73.4% could be further classified within the phylum Proteobacteria (Table S1), alongside classifications to the Bacteroidetes (7.7%), Nitrospirae (6.5%) and Planctomycetes (2.5%, Figure 5A). The remaining 9.9% of the sequencing reads were associated with 35 other Phyla which individually comprised <2.0% of the total community profile. Alpha diversity statistics for the sample can be seen in Table S2.

<Figure 5 here>

When reads were resolved to the genus level, a total of 828 separate genera were identified (Table S3). Of these, 51.7% of the community was comprised of genera that individually contributed to less than 2.0% of the total community structure (Figure 5B). Despite this, *Thiothrix* and *Thiobacillus* represented 16.2% and 8.7% of the community respectively. *Thiobacillus* and *Acidithiobacillus* have been noted within the microbial communities of a number of snottite biofilms, typically within acidic environments (Jones, Schaperdoth, and Macalady 2016). Both *Thiothrix* and *Thiobacillus.* also are cyclers of sulphur. *Thiothrix* has been shown to generate elemental sulphur ($S^0$) from sulphide ($HS^-$) or thiosulphate sources ($S_2O_3^-$, Ravin et al, 2022).

Of those reads associated with the genus *Thiothrix,* 10,426 could be further classified to show homology to *Thiothrix fructosivorans*. Previous studies have indicated that this species can form sheaths of carbohydrate (Kawasaki et al. 2017). These are thought to be a storage mechanism for sulphur granules and a protection mechanism from desiccation and metal oxide precipitation, but may also contribute to the materials associated with the snottite structure. The carbohydrate sheaths may also limit the diffusion of gases within the snottite, allowing for the generation of an oxygen limited environment. Microscopic investigation of the structure (Figure 6) suggested that the carbohydrate sheaths are present within the snottite and are similar in morphology to those previously described (de Graaff, van Loosdrecht, and Pronk 2020; Nielsen 1985). When homogenised with water, the pH of the sample was 4.8, which may suggest that there is biotic and/or abiotic production of sulphate (and subsequently sulphuric acid) within the core of the biofilm.

*Nitrospira* were the third most abundant genus of organism within the biofilm after *Thiothrix* and *Thiobacillus*. *Nitrospira* is an emerging genus of consideration with respect to its metabolic abilities. In particular, the species has been identified as having potential for complete ammonia oxidation (comammox) allowing for the conversion of ammonia to nitrate via nitrite, a reaction previously thought to be a multispecies process (Koch, van Kessel, and Lücker 2019). This may suggest that *Nitrospira* contribute to nitrogen turnover within the biofilm community. Further studies of *Nitrospira* sp. also

suggest the requirement for organic carbon (Bayer et al. 2021). Further study of the interactions between this organism would focus on their ability to consume the carbohydrate component of the biofilm.

Six OTUs represented between 2.4 and 2.8% of the total community structure. The sequences obtained for these OTUs came from uncultured collections and as such are typically given a designation based upon letter and numbers. These can be seen in Table 1 alongside the classification of these OTUs to the Family taxonomic classification through EzTaxon.

Interestingly, a single OTU associated with the Bacteriovoracaceae, comprised 2.4% of the community. The Bacteriovoracaceae are one of the five families of the Oligoflexia class, groups of organisms that have been described as obligate predators of bacteria (Ezzedine et al. 2020; Paix, Ezzedine, and Jacquet 2019). This suggests that the snottite community is not solely a symbiotic community of organisms, and that there are other microorganisms present that are opportunistically predating on the organisms within the biofilm material. There were further OTUs classified as Thiobacillaceae alongside organisms from the Sterolibacteriaceae, which have also been suggested to be involved with the cycling of sulphur compounds, linked to organic acids within hot springs. Example strains from the Sterolibacteriaceae family have been shown to grow at temperatures more representative of caves (0-25°C) (Watanabe et al. 2019).

The snottite-like biofilm that was obtained from Yatestoop sough displayed some similarities with respect to community profile in comparison to other snottites from acidic, subterranean locations. The presence of *Thiobacillus* and *Thiothrix* is similar to snottite community profiles found in Frassasi cave (Macalady et al. 2008) . In contrast, in those caves which are based in pyrite systems, iron oxidising genera such as *Leptospirillium* are found in greater abundance within the snottite community at the expense of *Acidithiobacillus/Thiobacillus* (Ziegler et al. 2009; Bond, Smriga, and Banfield 2000). Overall, the present study describes the bacterial community present within a snottite like biofilm from Yatestoop Sough. The results indicated that *Thiothrix* and *Thiobacillus* are the most numerous species within the bacterial community and that the community profile and formation of the snottite- like biofilms in Yatestoop Soughmay be influenced more by the elements available in the environment rather than bulk pH. Although only a single sample, the results here suggest that the biofilms at

Yatestoop Sough, and other subterranean environments merit further observation and may act as indicators for elemental shifts in water discharges.

**Acknowledgements**

The entrance to Yatestoop Sough is on private land and there is no public access. The owners, H.J. Enthoven & Sons, kindly granted permission to access for scientific sampling. The study was completed with the support of the Cave Science and Technology Research Fund (CSTRF).

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Table and Figure headings

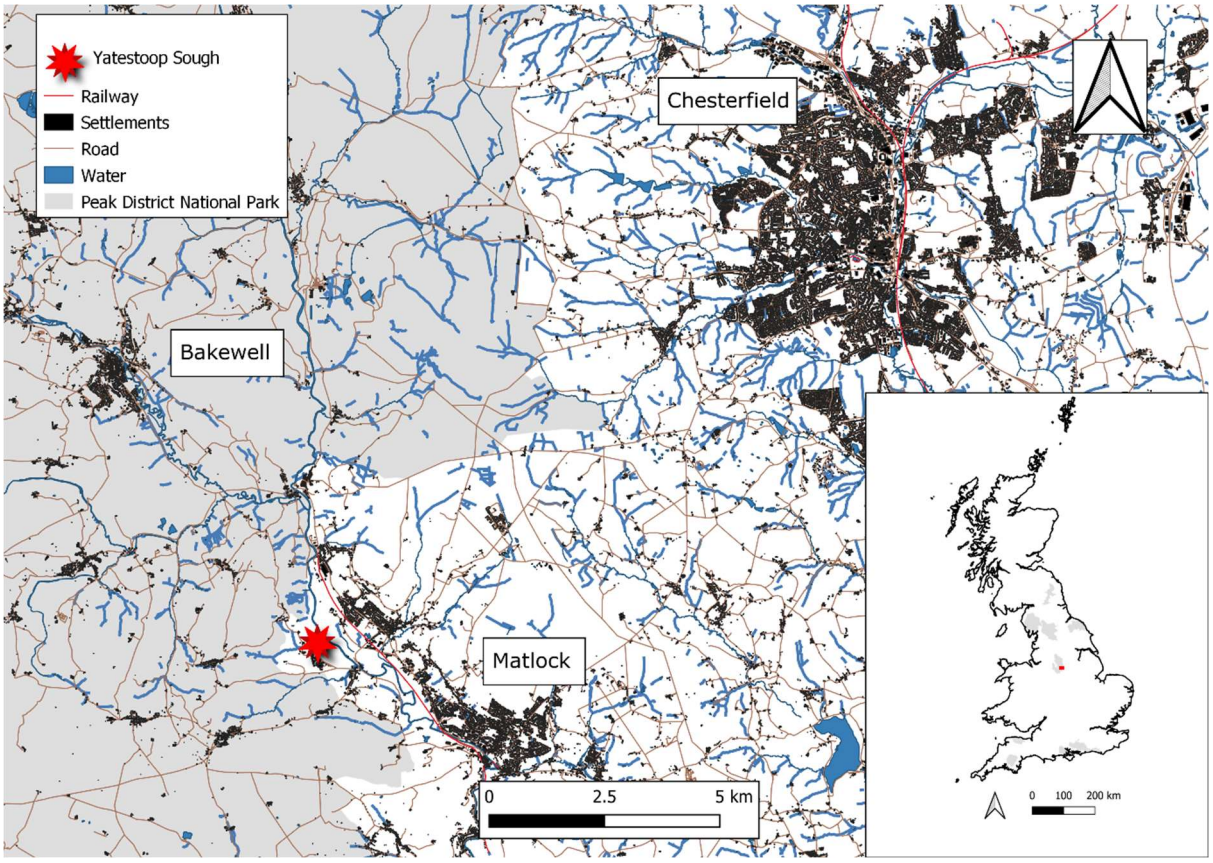


Figure 1: Map of sample site location.

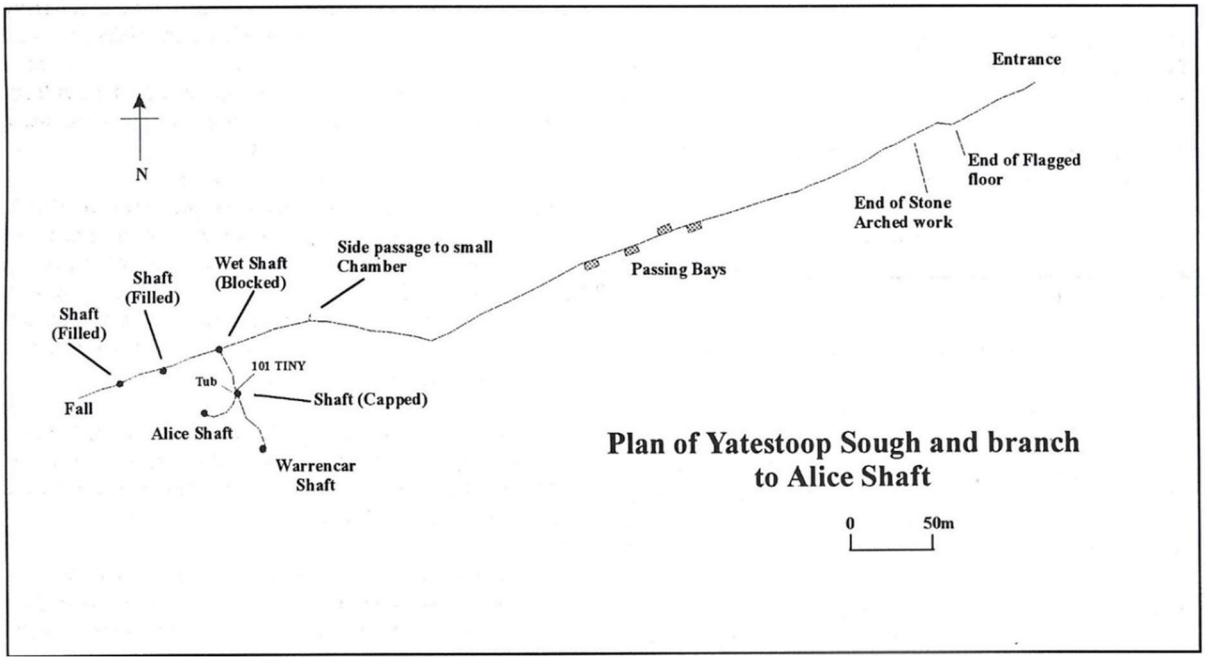


Figure 2: Plan of Yatestoop sough and branch to Alice Shaft

Figure 3: Images of mine structure and presence of biofilms in-situ.

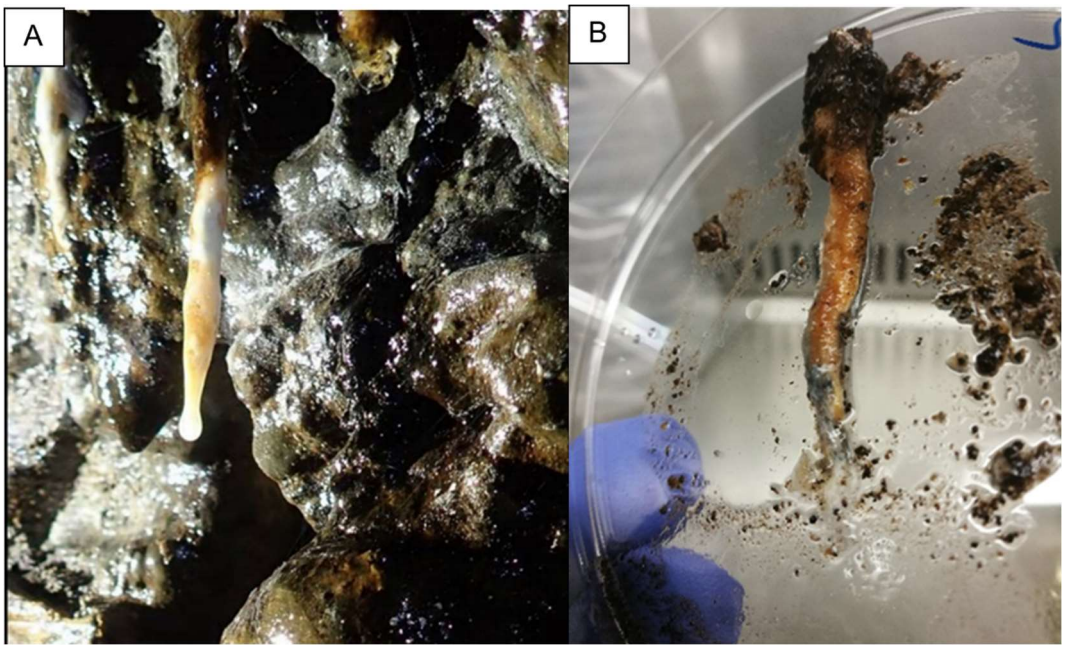


Figure 4: Snottite material obtained from sough wall in situ (A) and upon removal and return to laboratory (B) (photo credit: John Gunn (A) and Harry Mitchell (B))

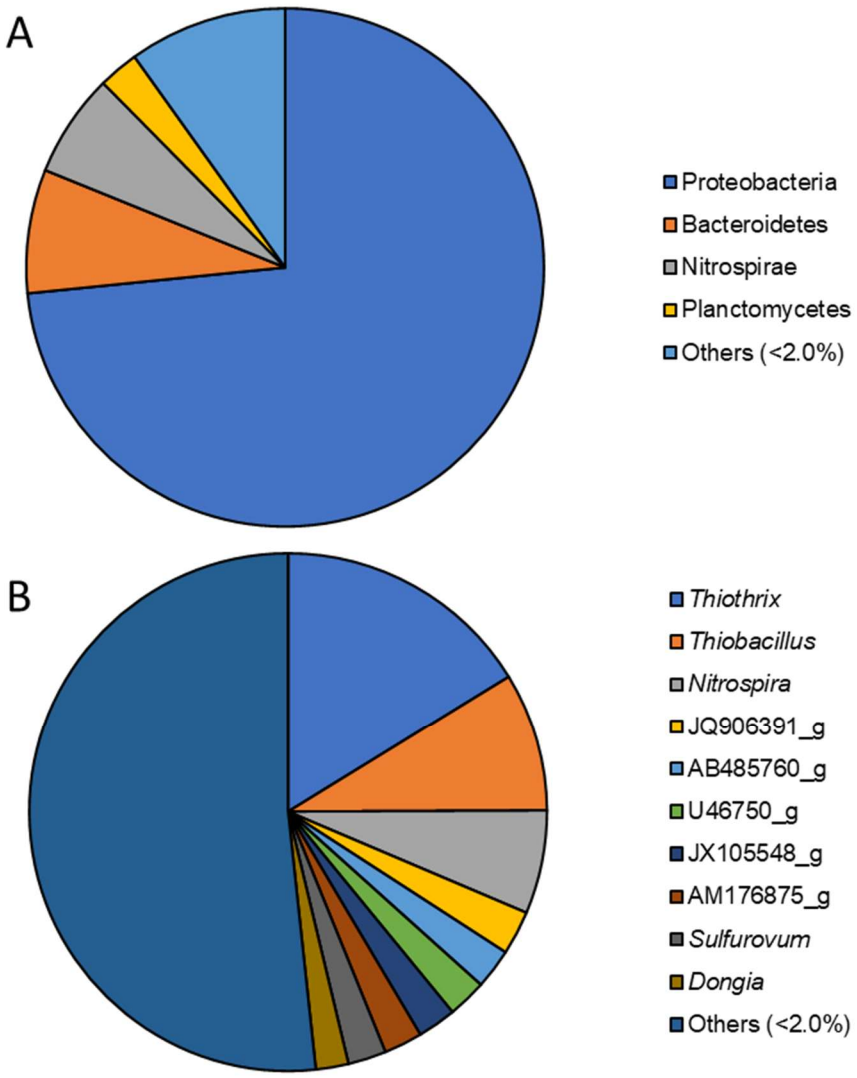


Figure 5: The taxonomic classification of 16S rRNA gene reads belonging to the Phylum (A) and Genus (B) classifications from the organisms within the snottite like biofilm.

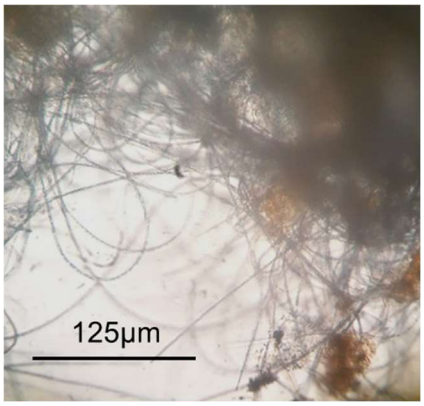


Figure 6: Microscopic investigation of the snottite

| Designation | Family |
|---|---|
| JQ96391_g | Methylomonas_f |
| AB485760_g | Sterolibacteriaceae |
| U46750_g | Thiobacillaceae |
| JX105548_g | Bacteriovoracaceae |
| AM176875_g | Chromatiaceae |

Table 1: Family designations of OTUs without a genus assignment.