University of South Bohemia

Faculty of Science

Stick together – effect of C, N source on extracellular polymeric compound producing bacteria from permafrost

Bachelor's thesis

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Annotation

The bacterial strains *Curtobacterium oceanisedimentum* and *Viridibacillus arvi* were incubated at different C/N ratios together with *Sphingomonas desiccabilis* as positive control. The Biochemical oxygen demand (BOD) was measured, the EPS quantified, the cells differentially stained and observed under a bright-field microscope, and the CFU ml^{-1} was determined.

Declaration

I declare that I am the author of this qualification thesis and that in writing it I have used the sources and literature displayed in the list of used sources only.

In České Budějovice,

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Acknowledgement

Abstract

The destabilisation of organic carbon (OC) in permafrost contributes to global warming. This can be prevented by extracellular polymeric substances (EPSs) by occlusion of OC in soil micro and macro aggregates. However, bacterial EPS production under changing environmental conditions, influencing both the vulnerability of soil organic carbon (SOC) stock and the functioning of microbial communities, is not fully understood. This study aimed to test different carbon-to-nitrogen (C/N) ratios on the production of EPS by *Curtobacterium spp.* and *Viridibacillus spp.* isolated from degraded permafrost soil. Both strains were incubated at C/N ratios of 4, 8 and 16 for 5 days at 25 ° C and 50 rpm and *Sphingomonas desiccabilis* as a positive control and the biochemical oxygen demand (BOD) was measured. The cells were then differentially stained and observed under 1,000x magnification. Furthermore, the strains were plated and the colony-forming units (CFU) ml^{-1} were determined. A decrease in BOD with increasing C/N ratio was found for *Curtobacterium oceanisedimentum* and *Viridibacillus arvi*. The EPS increased with increasing C/N ratio until C/N 8 for *Curtobacterium oceanisedimentum* and *Viridibacillus*. Differential staining and microscopy showed similar amounts and visibilities of capsules for the three strains. Only *Curtobacterium oceanisedimentum* shows a pronounced increase in CFU ml^{-1} from C/N 4 to C/N 8 and then a decrease from C/N 8 to C/N 16. The EPS produced clearly increased for *Curtobacterium oceanisedimentum* and *Viridibacillus arvi*. Both strains lack a trend in bacterial activity and growth; therefore, a wider variety of culture conditions, especially the C/N ratio, should be further tested.

List of abbreviations

Abbreviation	Meaning
C	Carbon
N	Nitrogen
kDa	Kilo Dalton = 1000 Dalton
DNA	Desoxyribonucleic acid
%	Percentage
°C	Degree Celsius
CPS	Capsular polysaccharides
SOC	Soil organic carbon
OC	Organic carbon
EPS	Extracellular polymeric substances
TB-EPS	Tightly bound Extracellular polymeric substances
M	Molar = mol litre^{-1}
mM	Millimolar = millimole litre^{-1}
OD$_{600}$	Optical density at 600 nm wavelength
Rpm	Rotations per minute
BOD	Biochemical oxygen demand
hPa	Hectopascal = 100 Pa
CFU	Colony forming units

1. Introduction

1.1 Extracellular polymeric substances (EPSs)

Extracellular polymeric substances (EPS), also called extracellular polysaccharides or exopolysaccharides, can protect microorganisms against high salinity (Nichols et al., 2004), antibiotic chemicals, and heavy metals, as well as prevent them from desiccation by retaining water within the EPS matrix surrounding the bacterium (Wingender et al., 1999b; Vardharajula and Ali, 2015; Wang et al., 2015). According to Morse (1990), EPSs are hygroscopic in nature, which might induce this water retention. In case of nutrient shortage, EPSs can also be metabolised to deliver energy and carbon (Sutherland, 2001a; Zhang and Bishop, 2003). EPSs are produced by microorganisms as a stress response in order to ensure survival (Nichols et al., 2005).

The properties of these molecules are significantly influenced by carbon source accessibility (Bhattacharjee et al., 2020), but also bacterial strains, cultivation time, substrate, and growth state (Read et al., 1987). Such compounds comprise large amounts of high molecular weight polysaccharides of about 10 – 30 kDa (Celik et al., 2008; Kumar et al., 2007), and in smaller amounts proteins, DNAs, lipids, and surfactants (Flemming et al., 2016; Flemming & Wingender, 2010; More et al., 2014). Microorganisms produce a wide variety of EPS (Schmid et al., 2015; Rehm, 2010), which can be classified into three categories. These are loosely bound EPS (LB-EPS), tightly bound EPS (TB-EPS), which stick to the cell as peripheral capsules, and amorphous slime (Comte et al., 2006).

This diversity in composition leads to even greater diversity in EPS properties and therefore functions (Chai et al., 2018). The composition of such microbial EPSs greatly changes under stress conditions (Konnova et al., 2001; Nandal et al., 2005).

1.2 Biosynthetic pathways of EPS

Nowadays, four different biosynthetic pathways for EPSs are known: the so-called Wzx/Wzy-dependent pathway, the ATP-binding cassette (ABC) transporter-dependent pathway, the synthase-dependent pathway, and the extracellular synthesis by use of a single sucrase protein. For the first three pathways, the monomers are enzymatically synthesised in the cell, following the same principle of forming activated sugars and sugar acids. On the

contrary, extracellular synthesis is achieved by sucrase adding sugar monomers to the forming polysaccharide (Schmid et al., 2015).

1.2.1 Wzx/Wzy-dependent pathway

In the Wzx/Wzy-dependent pathway, the monomers are built by glycosyltransferases and transported through the cytoplasmic membrane by a flippase enzyme (Wzx). In the periplasmic space, polymerisation takes place with the help of the polymerase (Wzy) enzyme, and the polymers are released onto the cell surface (Cuthbertson et al., 2009; Morona et al., 2009; Islam and Lam, 2014). This relief to the cell surface also depends on proteins linked to the polysaccharide co-polymerase (PCP) and the outer membrane polysaccharide export families (Cuthbertson et al., 2009; Morona et al., 2009). Polysaccharides synthesised via this Wzx/Wzy-dependent pathway are heteropolymers because they consist of different sugar molecules. Furthermore, the genes for the Wzx/Wzy enzymes are stored in the respective EPS operons (Schmid et al., 2015).

1.2.2 ATP-binding cassette (ABC) transporter-dependent pathway

The ATP-binding cassette (ABC) transporter-dependent pathway, which mainly occurs in capsular polysaccharide (CPS) biosynthesis, works similarly to the Wzx/Wzy-dependent pathway. Monomers are synthesised by one or several glycosyltransferases, later resulting in homo- or copolymers (Whitney and Howell, 2013). However, the transport through the cytoplasmic membrane is carried out by a tripartite efflux pump-like complex consisting of ABC-transporters and periplasmic proteins (Cuthbertson et al., 2009; Morona et al., 2009). The resulting polysaccharides have an attached glycolipid at the reducing terminus, which consists of phosphatidylglycerol and a poly-2-keto-3-deoxyoctulosonic acid (Kdo) linker (Willis and Whitfield, 2013; Willis et al., 2013).

1.2.3 Synthase-dependent pathway

In the synthase-dependent pathway, polymerisation takes place within the cell and therefore complete polysaccharide strands are relieved through the cell wall and membrane. Both polymerisation and translocation are performed by enzyme synthase, which is for some compounds a subunit of a multiprotein complex (Rehm, 2010). The synthase-dependent pathway strictly produces homopolymers, although several preliminary homopolymers

consisting of different monomers can be connected to form block polymers (Rehm and Valla, 1997). An exception is the biosynthesis of hyaluronic acid, which forms copolymers with the help of hyaluron synthase (Chong et al., 2005).

In summary, monomer/dimer synthesis mostly takes place within the cell, while polymerisation and secretion into the extracellular space occur in the periplasmic space. However, these biosynthetic processes of EPSs are currently not entirely understood (Schmid et al., 2015).

The genes for polymerising, branching, substituent-adding, and monomer modifying enzymes, as well as glycosyltransferases are contained in either the genomic or plasmid DNA of most of the EPS-producing microorganisms (Finan et al., 1986; Rehm, 2010).

1.3 Industrial use of EPSs

Some of these EPSs are applied in industry for their structural or physical properties, leading also to a financial interest in the research of these compounds (Chawla et al., 2009; Rehm, 2009; Imeson, 2010). For example, EPSs are added to food products to mix them better or to manipulate their haptic properties (Jurášková et al., 2022). Furthermore, these compounds are used in medicine, acoustics (Ross et al., 1987; Rehm, 2010), oil industry, cosmetics (Glucksmann et al., 1993b; Becker et al., 1995a) and more applications. These compounds can be harvested cheaply and continuously from the culture supernatant previously separated from cells (Schmid et al., 2015).

1.4 Soil aggregation by EPSs

On the other hand, EPSs can also form microbial aggregates (Wingender et al., 1999b; Vardharajula and Ali, 2015; Wang et al., 2015; Sheng et al., 2010; Martens, 1993, which can adhere to surfaces and aid in soil particle cementation. This cementation is possible because of cation bridges, hydrogen bonds, Van der Waals forces, anion adsorption mechanisms, and the slimy surface of some EPSs. These help the microbial aggregates to tightly hold onto clay and ionic particles (Vardharajula & Sk Z, 2014; Chenu, 1995; Tisdall & Oades, 1982. The stability of these aggregates might be further improved by the reduction of wettability and swelling (Hillel, 1982). The EPSs occur in fibres that connect clay particles and therefore form an area of clay and EPSs that is surrounded by soil. The structures formed are

then called microaggregates (<250 μm) and macroaggregates (>250 μm) (Sandhya & Ali, 2015). Especially microaggregates stabilise SOC within the soil, providing longer lasting storage of carbon (Smith et al., 2014; Wiesmeier et al., 2019). Aggregates are also important for soil texture which influences biological mechanisms in the soil. Therefore, they keep and move water many times their weight at low water potentials (Sandhya & Ali, 2015), acquire nutrients (Vicente-Garcia, 2004), provide oxygen, reduce runoff, and mediate the temperature. This is done with the help of pores that originate from the reorganising, flocking, and cementing of soil particles (Alami et al., 2000; Tang et al., 2011). This cementing of soil particles by EPSs could also have a role in stabilizing SOC in degraded permafrost soil, which is the high reservoir of OC.

1.5 Permafrost soil as a storage for organic carbon (OC)

Permafrost is the soil, the temperature which has perennially been below 0 °C for at least two consecutive years. It mainly appears at the poles or beneath huge glaciers. Permafrost can occur as dry frozen ground, when the soil does not contain any water, or as wet frozen ground, when the soil contains liquid water. Even if the permafrost is wet, the water might not be frozen, mainly due to the high pressures below the thick glaciers or the high salinity of the sea water, which is true for the continental shelf of Alaska. This permafrost is then referred to as cryotic (Dobinski, 2011).

The total soil organic carbon (SOC) stored in the permafrost soil of the northern hemisphere is 1,460 to 1,600 Pg (Schuur et al., 2022) and therefore more than the total carbon stored in the atmosphere (Hugelius et al., 2014; Schuur et al., 2015). Mishra et al. (2021) pointed out that the SOC was highest between the mean annual temperature of -10 and -5 ° C and the mean annual precipitation of 200 and 600 mm.

1.6 Permafrost Degradation

When permafrost thaws due to an increase in atmospheric temperature, this SOC becomes available for heterotrophic microorganisms, which releases it into the atmosphere in the form of CO_2, CH_4 or N_2O reinforcing global warming (Schuur et al., 2015; Zimov et al., 2006; Schuur et al., 2008; Anisimov et al., 1999; Mueller et al., 2015; Nelson et al., 2001; Prater et al., 2020; Schuur et al., 2008; Ro et al., 2008; Zimov et al., 2006). This thawing deepens

the active layer (Anisimov et al., 1999; Mueller et al., 2015; Nelson et al., 2001; Prater et al., 2020; Schuur et al., 2008), providing more oxygen, nutrients and microorganisms to the soil (Lawrence et al., 2015; St Jaques & Sauchyn, 2009); Schuur and Mack, 2018). These processes can promote the degradation of deeper SOC (Fontaine et al., 2007; Gocke et al., 2010; Schuur et al., 2015; Wild et al., 2016).

For a specific location in the Yedoma Ice Complex in north-eastern Siberia, 70 % of the carbon stored in the permafrost is outgassed into the atmosphere within 120 years when assuming present-day climate conditions (Khvorostyanov et al., 2008).

Another study compared more than 100 locations in the Arctic and suggested that 1600 Tg C per winter are outgassed by permafrost soils (Natali et al., 2019). Furthermore, since permafrost degradation is not linear, huge amounts of SOC can reach both the atmosphere and the ocean in a very short time (Jin & Ma, 2021).

1.7 Factors influencing EPS Production

As EPS can have an impact on SOC aggregation and therefore the storage of such C, it is important to know which factors mainly influence their formation. EPS production levels depend on several factors; some of the most influential are the carbon source and the C/N ratio. In general, it can be said that a higher C/N ratio increases EPS production (Magaritis & Pace, 1986; Lee et al., 2007). In recent studies, the highest level of EPS production has been observed with glucose as a carbon source, which is easily degradable and probably the precursor used most for EPS production and a ratio of 13.6 g C / g N (Miqueleto et al., 2010).

1.8 Differential staining

When secreted in the environment of the cell, tightly bound EPS appear as capsular polysaccharides (CPS) that are attached to the cell wall. Charged capsules can bind large amounts of water, which can lead to deformation of the capsular structure during the staining procedure (Bayer, 1990). The staining method should be well selected for each strain, as the structural diversity of EPS capsule could interact with the stain. Fixing and drying of cells might cause the contraction and even destruction of the capsules (Duguid, 1951). While advanced techniques such as electron microscopy are increasingly used for the visualisation

of such CPS, light microscopy remains an easy and cheap technique that is widely available (Oleksy & Klewicka, 2017).

1.9 Strains of interest and aims

For this experiment, strains of psychrophilic bacteria that have previously been isolated from degraded permafrost soil located in central Alaska were used. These strains were selected for this study based on their EPS production determined by an automated analytical system including various polysaccharide detection modules at Technische Universität München (TUM) Campus Straubing, Germany. The first strain was *Curtobacterium oceanisedimentum*, which produced up to 217 mg l^{-1} EPS from glucose. This strain occurs as creamy, yellow or orange colonies with a diameter of 1 to 1.5 mm or more than 2 mm, depending on the strain. The colonies of some *Curtobacterium spp.* have shiny and slimy surfaces and grow well in a temperature range of 30-37 ° C. *Curtobacterium spp.* appear in a coryneform shape, are gram-positive and motile and possess an oxidative metabolism (Funke et al., 2005). The second strain was *Viridibacillus arvi*, which produced about 100 mg l^{-1} EPS from glucose. This strain appears in even round-ended rods found both singly and in pairs. This strain grows in a temperature range of 15 to 37 ° C, growing best at 28 ° C. The growth also takes place in a pH range from 6.0 to 9.0, with best growth at pH = 7.5. Some *Viridibacillus spp.* occur as cells that are motile, gram-positive and sporulating, forming relatively round endospores (Xu et al., 2021). *Sphingomonas desiccabilis* (CBR S 236 DSMZ 16792), which produces EPS, was used as a positive control obtained from TUM Campus Straubing, Germany.

The aim of this experiment was to test different amounts and ratios of carbon and nitrogen on the production of extracellular polymeric substances by *Curtobacterium spp.* and *Viridibacillus spp.* isolated from degraded permafrost soil.

2. Materials and Methods

2.1 Preparation of solutions

The EPS medium is easily contaminated, as it provides the basic nutrients crucial for microbial growth. Therefore, to avoid contamination, partial solutions 1-3 were autoclaved, the trace element solution was filtered with a 0.22 µm syringe filter, and all steps after autoclave were carried out in a laminar flow hood close to a flame. Three EPS media with C/N ratios of 4, 8 and 16 were prepared to test different C/N ratios on EPS production. For the agar medium used for streaking of bacteria, 350 ml was prepared, for the pre-culturing 250 ml and for the incubation experiment 700 ml of EPS medium were prepared each time they were needed. The EPS medium proposed in Rühmann et al. (2015), used in this experiment in general consists of solutions 1-5. Solution 1 contains 1.4 $\frac{g}{l}$ peptone from casein, 1.2 $\frac{g}{l}$ MgSO4·7 H_2O, 9.0 $\frac{g}{l}$ agar (only for petri dishes). Solution 2 contains 0.04 $\frac{g}{l}$ $CaCl_2$·2 H_2O, different amounts of D-$C_6H_{12}O_6$·H_2O (Table 3). Solution 3 contains 1.5 $\frac{g}{l}$ KH_2PO_4, solution 4 0.2 % of vitamin solution (Sigma R7256) and solution 5 consists of 0.1 % trace element solution. The trace element solution consists of 1.8 $\frac{g}{l}$ $MnCl_2$·4 H_2O , 2.5 $\frac{g}{l}$ $FeSO_4$·7 H_2O pure, 0.258 $\frac{g}{l}$ H_3BO_3, 0.031 $\frac{g}{l}$ $CuSO_4$·5 H_2O pure, 0.021 $\frac{g}{l}$ $ZnCl_2$ pure, 0.075 $\frac{g}{l}$ $CoCl_2$·6 H_2O, 0.023 $\frac{g}{l}$ Na_2MoO_4·2 H_2O and 2.1 $\frac{g}{l}$ $NaKC_4H_4O_6$ (Table 2). The chemicals were obtained from Lach:Ner (Neratovice, Czech Republic), except for peptone and vitamin solution, which were obtained from SIGMA-ALDRICH (Darmstadt, Germany), and the agar, which was obtained from OXOID. For preparation, several partial solutions were prepared, two of them containing the following chemicals: Peptone, magnesium sulphate heptahydrate, and – if needed – agar, as well as potassium dihydrogen phosphate, respectively. For these two partial solutions, the pH was adjusted using a pH metre (SCHOTT pH-METER) and 4 M sodium hydroxide solution provided in the laboratory. The total C content in the mixed partial solutions 1, 3, 4 and 5 was previously determined and amounts to 0.520 $\frac{g}{l}$, which results in an initial C/N ratio of 3.6, even before adding solution 2. To achieve C/N ratios of 4, 8.5 and 16, 0.13 g, 1.95 g and 4.22 g $C_6H_{12}O_6$·H_2O were added for a final volume of the combined medium (solutions 1-5) of 700 ml, respectively (Table 3). Equation 1 was used for this calculation.

$$\left(\frac{glucose\ monohydrate\ added \cdot volume\ used \cdot ratio\ C\ in\ glucose\ monohydrate}{diluted\ in\ volume \cdot final\ volume\ combined}\right.$$

$$\left. + \ initial\ C\ content\right) : initial\ C\ content \cdot initial\ C{:}N\ ratio$$

$$= \left(\frac{0.13 \cdot 0.056 \cdot 0{,}3636813234}{0.08 \cdot 0.7} + 0.52\right) : 0.52 \cdot 3.6. \qquad \textit{Equation 1}$$

This addition resulted in a glucose monohydrate concentration in the final combined EPS medium of 0.19, 2.79 and 6.0 $\frac{g}{l}$ as shown in Table 3. To sterile solutions 1-3, they were autoclaved separately for 20 minutes at 121 ° C. Then exactly 2 ml of vitamin solution (R7256 Sigma-Aldrich) were pipetted and 1 ml of a trace element solution was filtered with a 0.22 µm syringe filter (Schoeller pharma, Prague, Czech Republic). Both solutions were added to the EPS medium in a laminar flow hood.

A 0.9 % saline solution was prepared by dissolving 3.15 g of NaCl (Lach-Ner) in 350 ml of deionised water. A 10 mM NaOH solution was prepared by dissolving several NaOH beads corresponding to 0.16 g in 40 ml of deionised water, and then dissolving 5 ml of that solution in 45 ml deionized water. A 20 % copper sulphate solution was prepared by dissolving 10 g $CuSO_4 \cdot 5\ H_2O$ in 50 ml of deionised water.

Table 1 Composition of the EPS medium.

Partial solution nr.	Compound	Concentration in the combined medium in $\frac{g}{l}$ if not differently stated	Note
Solution 1	Peptone from casein	1.4	pH adjusted to seven, autoclaved separately
	MgSO4·7 H_2O	1.2	
	Agar	9.0	
Solution 2	$CaCl_2$·2 H_2O	0.04	Autoclaved separately
	$C_6H_{12}O_6$·H_2O		
Solution 3	KH_2PO_4	1.5	pH adjusted to seven, autoclaved separately
Solution 4	Vitamin solution	0.2 %	
Solution 5	Trace element solution	0.1 %	Sterile filtered

Table 2 Composition of the trace element solution.

Trace element	Concentration in the partial solution 5
$MnCl_2 \cdot 4\ H_2O$	1.8
$FeSO_4 \cdot 7\ H_2O$	2.5
H_3BO_3	0.258
$CuSO_4 \cdot 5\ H_2O$	0.031
$ZnCl_2$	0.021
$CoCl_2 \cdot 6\ H_2O$	0.075
$Na_2MoO_4 \cdot 2\ H_2O$	0.023
$NaKC_4H_4O_6$	2.1

Table 3 Amounts of glucose monohydrate added in the three incubation experiments, and the resulting concentrations of glucose monohydrate and C, as well as the final C/N ratio.

Incubation experiment nr.	Mass of $C_6H_{12}O_6 \cdot H_2O$ added to a final volume of 700 ml in g	Concentration of $C_6H_{12}O_6 \cdot H_2O$ in the combined medium in $\frac{mol}{l}$	Concentration of C added in $\frac{g}{l}$	Concentration of total C in $\frac{g}{l}$	Final C/N ratio
1 (C/N 4)	0.13	0.19	0.047	0.57	4
2 (C/N 8)	1.95	2.79	0.709	1.23	8
3 (C/N 16)	4.22	6.0	1.534	2.05	16

2.2 Bacterial strains

Strains from *Curtobacterium oceanisedimentum* and from *Viridibacillus arvi* were previously isolated from permafrost soil in Fairbanks, Alaska, USA, in 2021. *Sphingomonas desiccabilis* (CBR S 236 DSMZ 16792) was obtained as a positive control. The three

bacteria were aseptically taken with an inoculation loop and streaked on EPS agar with the streak plate technique and incubated for three days at 22 ° C.

2.3 Pre-culture preparation

The partial solutions (as described in the subsection 'Preparation of solutions") were combined in a laminar flow hood close to a flame to avoid contamination. 20 ml of this combined EPS medium was pipetted into a 50 ml Falcon tube. Then a well-grown colony from the agar plate was aseptically taken with an inoculation loop and transferred to the Falcon tube. This procedure was repeated for all three bacterial strains. Additionally, a fourth Falcon tube was also filled with 20 ml of EPS medium, but without adding any bacterial culture. This fourth Falcon tube served as a negative control to ensure the absence of any contamination. The Falcon tubes were incubated on a shaker for 36 hours at 22 ° C and 200 rpm.

2.4 Washing of cells

Where possible, the following steps were carried out in a laminar flow hood to avoid contamination. The 100 µl liquid culture of each Falcon tube were transferred onto EPS agar Petri dishes by streak plate technique to confirm the purity of the cultures. Petri dishes were incubated at 22 ° C for 2 days. Subsequently, the liquid culture of each Falcon tube was distributed in ten 2 ml Eppendorf tubes. One of them was used to measure the optical density at 600 nm (OD_{600}) of each liquid culture, to be able to add the same amount of bacterial biomass for each bacterial strain to the incubation experiment. The remaining nine Eppendorf tubes were centrifuged (MIKRO 120 and 185: Hettich GmbH and Co. Germany) at 13000 rpm for ten minutes and the supernatant was cautiously discarded to not discard any bacterial biomass. To five Eppendorf tubes, 2 ml of 20 % glycerol solution was added, and they were stored at -20 ° C for later use. The remaining four Eppendorf tubes were used to add the bacterial biomass to the EPS experiment; therefore, 2 ml of 0.9 % saline solution was added to them. Subsequently, the tubes were centrifuged again at 13000 rpm for ten minutes and the supernatant was discarded. Then, to the four Eppendorf tubes, 2 ml of 0.9 % saline solution were added and they were vortexed until the bacteria pellet was entirely detached from the bottom and dispersed in the saline solution and the OD600 was adjusted to 1.

2.5 Storage of bacteria

Bacterial cultures on EPS agar were stored at 4 ° C. Liquid bacterial cultures in 0.9 % saline solution were stored at 4 ° C. Liquid bacterial cultures in 20 % glycerol solution were stored at -20 ° C for use within the next few weeks and at -80 °C for resetters to be used at any later time.

2.6 Incubation

Three incubation experiments were carried out to test the impact of a C/N ratio of 4, 8 and 16 on EPS production. To avoid contamination, the following steps until incubation were carried out in a sterile microbial workbench. Furthermore, the following materials were autoclaved for 20 minutes at 121 ° C for each C/N ratio, respectively: 12 250 ml reagent bottles, twelve containers for OxiTop measuring systems (WTW OxiTop®-C), 5 ml tips, 0.1 M sodium hydroxide solution, saline solution and the three partial solutions consisting of peptone and magnesium sulphate heptahydrate, the second consisting of calcium chloride dihydrate and glucose monohydrate, and the third containing potassium dihydrogen phosphate. In the sterile microbial workbench, 50 ml of the combined EPS medium was pipetted into three 250 ml reagent bottles each. Immediately after, 1 ml of an $OD_{600} = 1$ bacterial solution from the pre-culturing was added to the respective bottle. This step was repeated for the three bacterial strains. The negative control consisted of only 50 ml of the EPS medium. The incubation reagent bottles were in triplicate for each strain for each C/N ratio. 100 µl of 10 mM sodium hydroxide solution was pipetted into the OxiTop containers and screwed into the measuring system. The OxiTop measuring systems for respiration were then screwed on the reagent bottles, and the biological/biochemical oxygen demand (BOD) measurement was started. The bottles were then incubated on a shaker at 50 rpm and 25 ° C for five days. Low rpm was used to prevent the NaOH solution from spilling into the bacterial solution. After incubation the pressure (in hPa) obtained from the OxiTop systems were converted to BOD (in mmol l^{-1}) with Equation 2. The BOD in mmol l^{-1} was further converted to the BOD in mg l^{-1} by multiplying with the molar mass of molecular oxygen ($M(O_2) = 32$ g mol^{-1}). As the stoichiometry of O_2 and CO_2 during cellular respiration is 1:1, the amount of substance of the CO_2 produced equals the amount of substance of the oxygen

consumed. Therefore, to convert the BOD in mg l^{-1} to the CO_2 in mg l^{-1}, multiplication with the ratio of the molar masses of CO_2 and O_2 had to be conducted: $\frac{M(CO_2)}{M(O_2)} = \frac{44}{32} = 1.375$

$$BOD = absorption(O_2) \cdot 100 \cdot (\frac{V_{headspace} - V_{bac.\ sol.}}{(T+273.15) \cdot R \cdot V_{bac.\ sol.}}) \text{ [mmol l}^{-1}]$$ *Equation 2*

With absorption(O_2) being the pressure decrease measured by the OxiTop systems, the temperature being in °C, and R being the gas constant.

2.7 EPS determination

After the incubation 45 ml of liquid cultured were transferred to 50 ml Falcon tubes. the liquid culture was shear-treated to separate the EPS from cells by UltraTurrax T25 with N25G-18G dispersing tool (autoclaved). Afterward, the liquid cultures were centrifuged at 8500 g to separate the suspended EPS (supernatant) from the cells (pellet). The supernatant was transferred into fresh sterile falcon tube and sent to the Institute of Soil Science and Site Ecology, Technical University of Dresden, Germany. Where they have carried out some procedures such as desalination via dialysis and freeze-dried method to extract the EPS from the supernatant.

2.8 Differential Staining of capsules and Microscopy

To avoid contamination of the samples through the environment, the procedures were carried out as close to a flame.

2.8.1 Anthony's method

Anthony's method (Oleksy & Klewicka, 2017) was used to make the bacterial cells and the surrounding EPS capsules observable under a bright field microscope (Olympus BX61, Japan) by differential staining. Therefore, 20 µl of mixed bacterial solution were pipetted on a microscope slide (Knittel Glasbearbeitungs GmbH, Braunschweig, Germany), which was previously cleaned with ethanol, and dispersed with another such slide. After air-drying without flame fixing the bacterial smear was stained with a 1 % crystal violet solution for

two minutes (Table 4). Then, the microscopy slide was washed with 20 % copper sulphate solution and air-dried. Finally, the smear was observed under immersion in oil at 1,000x magnification.

2.8.2 Hiss's method

Hiss's method was additionally used for differential staining, as bacterial cells and capsules were very hard to see (Oleksy & Klewicka, 2017). Thus, 20 µl of mixed bacterial culture were transferred onto a microscope slide cleaned with ethanol and dispersed with another slide. Secondly, the bacterial smear was cautiously flame-fixed, stained with 0.1 % crystal violet, and cautiously heated until steaming for approximately one minute. The slide was washed with 20 % copper sulphate, air-dried and observed under oil immersion at 1,000x magnification.

2.8.3 Nigrosin staining

Thirdly, nigrosine staining was conducted, as well to get a wider variety of microscopy pictures. For this staining method, 20 µl of mixed bacterial culture were also transferred onto a microscope slide cleaned with ethanol and dispersed with another slide. The smear was air-dried, stained with 10 % diluted nigrosine stain (Sigma Aldrich GmbH), again air-dried, and examined under oil immersion at 1,000x magnification. Table 4 compares the three differential staining methods.

Table 4 Comparison of capsule staining methods.

	Anthony's	Hiss's	Nigrosine
Reagents	1 % crystal violet 20 % copper sulphate	0.1 % crystal violet 20 % copper sulphate	10 % nigrosine
Bacterial drying	Air-dry	Flame-fix	Air-dry
Stain soaking time	2 min	Until steaming/1 min	Until air-dried

Immediately after differential staining, the microscopy slides were examined under the microscope with oil immersion at 1000x total magnification. Several dozen pictures were taken by the camera (Canon DS126571) attached to the microscope for each slide to get a more representative image of the capsules.

2.9 CFU

To control the purity of the nutrient medium and the 0.9 % NaCl solution, a Petri dish with EPS medium was incubated without anything and only with the NaCl solution, respectively. Next, a dilution series of each sample was carried out to a concentration of 10^{-6} compared to the original bacterial solution from the pre-culture preparation. Therefore, 100 μl of each sample was added to 900 μl of 0.9% NaCl and the resulting dilution was used for further dilution. This procedure was repeated six times until a dilution of 10^{6} was obtained. The solutions were vortexed before each dilution step to ensure a uniform distribution of the bacteria. 100 μl of solutions with concentrations 10^{-4}, 10^{-5} and 10^{-6} were pipetted on an EPS agar plate each and evenly spread on the plate with a sterile glass rod. and incubated at 25 ° C. After 5 days, the colony-forming units (CFU) were determined by using the following equation.

$$CFU = \frac{number\ of\ colonies\ on\ the\ plate \cdot diltution\ of\ the\ sample}{plating\ volume}\ [ml^{-1}] \qquad \textit{Equation 4}$$

3. Results

3.1 Pre-culture preparation and washing of cells

The negative control was a relatively clear and transparent liquid, indicating a lack of bacterial growth. The samples, including *Sphingomonas desiccabilis, Curtobacterium oceanisedimentum,* and *Viridibacillus arvi,* grew well during the incubation time. These findings confirmed an aseptic procedure and sufficient bacterial growth. The Petri dishes showed the same bacterial growth as the initially used colonies, which confirmed the purity of the pre-culturing.

3.2 Incubation

The negative control appeared as a relatively clear and transparent liquid, which is the EPS medium added that shows no growth of bacteria (Fig. 1A). Therefore, this confirms that the procedure was sterile, as planned by autoclaving the medium and using a laminar flow hood. However, the control in the first attempt of the third incubation experiment was turbid, indicating contamination of the EPS medium. Reagent bottles inoculated with *Sphingomonas desiccabilis* appeared as yellow-brown turbid liquid, indicating the growth of bacteria (Fig. 1B). Reagent bottles inoculated with *Curtobacterium oceanisedimentum* also appeared as yellow-brown turbid liquid, only a step lighter, indicating the growth of bacteria (Fig. 1C). The reagent bottles inoculated with *Viridibacillus arvi* appeared as grey, only slightly yellow-brownish coloured relatively turbid liquid, indicating the growth of different bacteria (Fig. 1D).
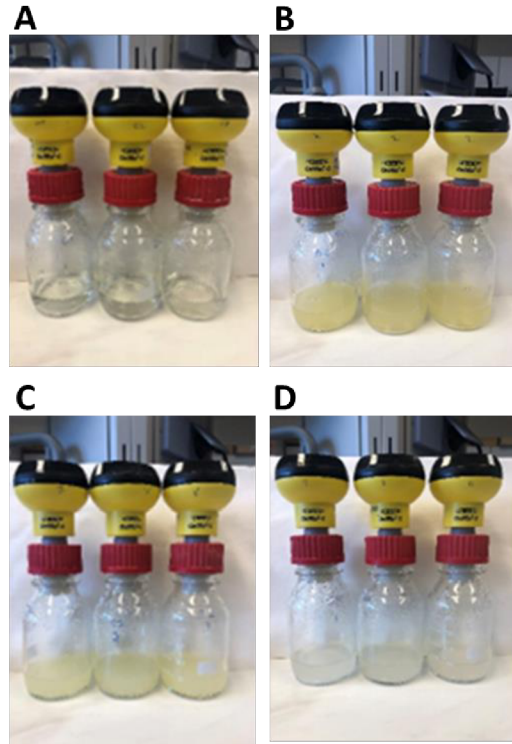
Figure 1 Bacterial growth after 5 days of incubation in EPS medium, (A) control, (B) Sphingomonas desiccabilis, (C) Curtobacterium oceanisedimentum, and (D) Viridibacillus arvi.

3.3 Respiration rate during incubation

OxiTop measurement systems provided pressure changes in hectopascal (hPa) during incubation time, by measuring the pressure within the tightly closed bottle every 20 minutes. Due to contamination, which could be seen as an acute and very pronounced decrease in pressure, a bottle from the negative control from the second experiment and a bottle containing *Viridibacillus arvi* from the third experiment were excluded from further analysis. Furthermore, BOD values of control samples were subtracted from the BOD values of stains samples. The BOD from 90 hour to 115 hour of incubation was averaged to obtain the O_2 consumption mentioned in Figure 2. The BOD of the positive control *Sphingomonas desiccabilis* decreased from 250 to 157 and further to 148 mg l^{-1} with increasing C/N ratio. In contrast, the BOD of the sample strains *Curtobacterium oceanisedimentum* and *Viridibacillus arvi* changed from 256 to 159 and further to 146 mg l^{-1} and increased from 353 to 423 and decreased again to 336 mg l^{-1}, respectively (Figure 2).

The respiration rate during the incubation was varied for each strain among the C/N ratio. the respiration rate was high during the C/N ratio 4 compared to C/N ratio 8 and 16 (Figure 3) Among the strains the respiration rate was higher for the *Curtobacterium oceanisedimentum,*

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up to 600 mg/l for all three C/N ratios, while for *Viridibacillus arvi* and *Sphingomonas desiccabilis* was approximately 400 mg/l in C/N 4 and 300 mg/l in C/N 8 and 16 (Figure 3).
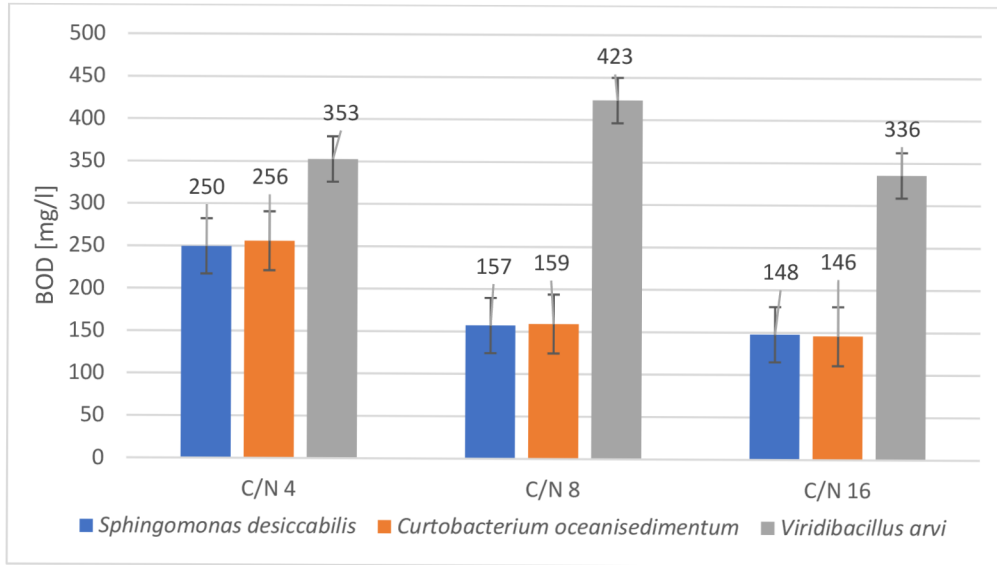

Figure 2 BOD in mg l⁻¹ after subtracting the values of the negative control.
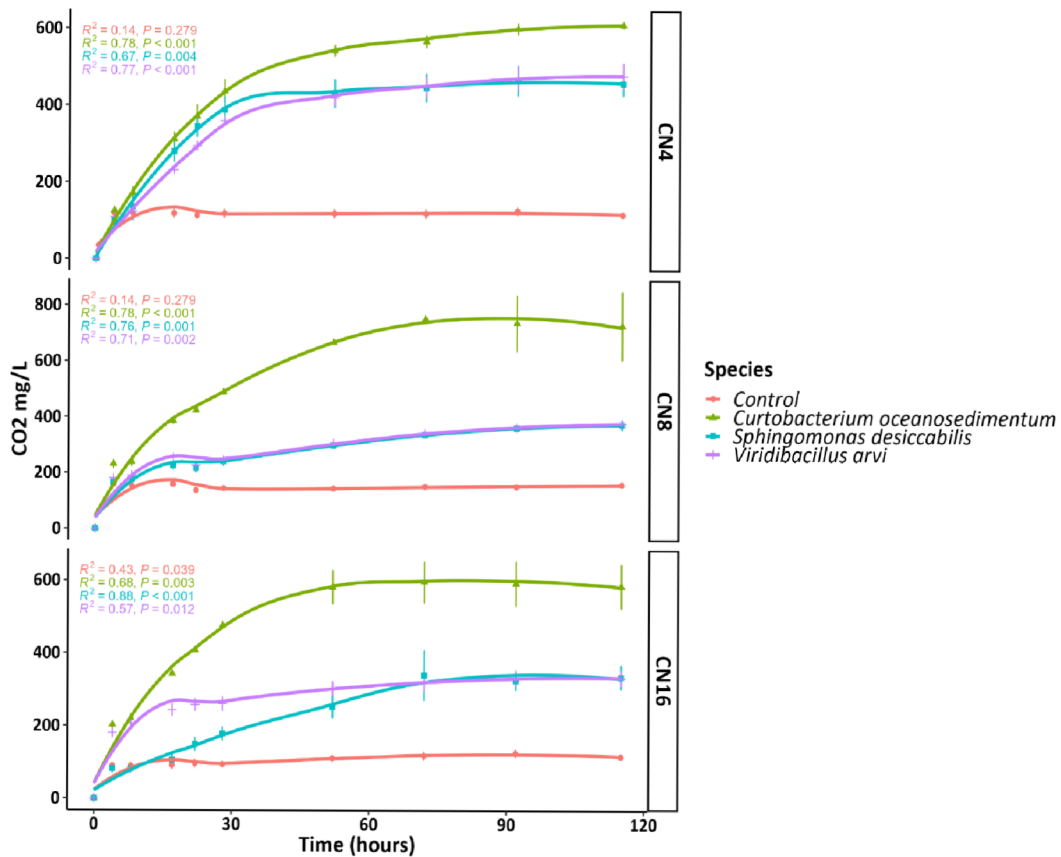

Figure 3 Respiration rate for the different bacterial strains from the incubation experiment.

3.4 EPS determination

We have received the data of extracted EPS from the supernatant we have sent to Institute of Soil Science and Site Ecology, Technical University of Dresden, Germany. The extracted EPS as shown in Figure 14 are in grams after subtracting from the control samples. According to this data, the EPS production by permafrost soil bacteria and the control was higher for C/N 8 than for C/N 4 for *Curtobacterium oceanisedimentum* and *Viridibacillus arvi* but did not further increase in C/N 16. The produced EPS by *Curtobacterium oceanisedimentum* (25 g in C/N 8 and C/N 16 and *Viridibacillus arvi* was high.
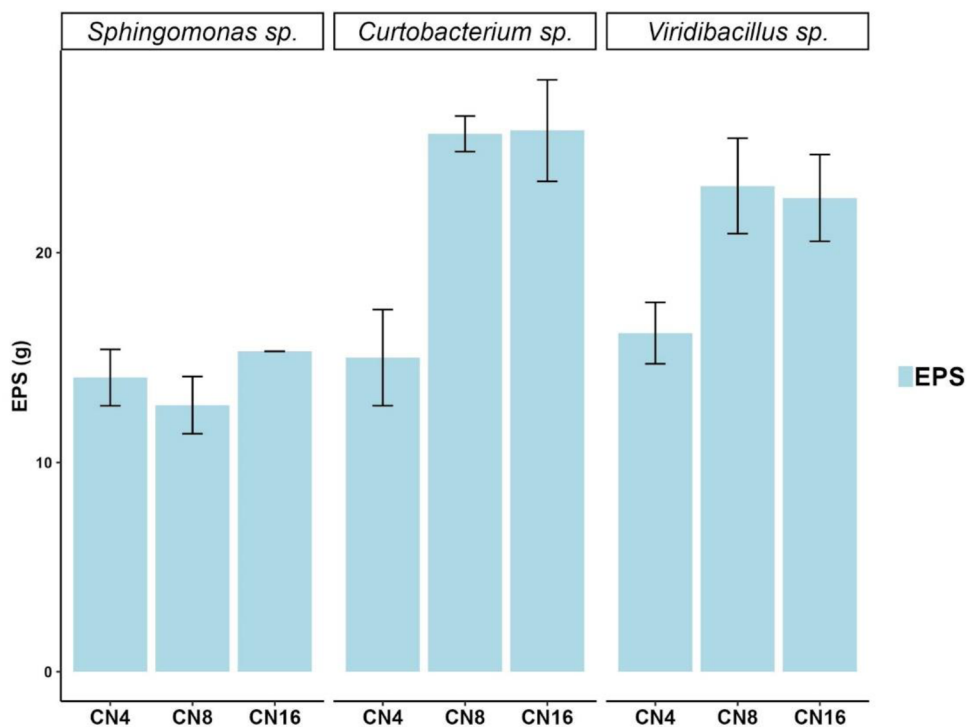

Figure 4 EPS production by strain under different C/N ratio.

3.5 Differential Staining of capsules and Microscopy

Figures 5-7 show the differentially stained bacteria from experiment 1 (C/N 4). Figures 8-9 show the differentially stained bacteria from experiment 2 (C/N 8). Figures 10-12 show the differentially stained bacteria from experiment 3 (C/N 16). *Sphingomonas desiccabilis* could be seen as violet/black stained spheres on a brighter background. *Viridibacillus arvi* and *Curtobacterium oceanisedimentum appeared* similarly as rods. The same shape of the bacteria on each slide indicated the purity of the bacterial solution. The EPS capsules appeared as white (unstained) rings around the bacterial cells (the arrows point on the capsules) (Oleksy & Klewicka, 2017), while the cells and the background were stained dark

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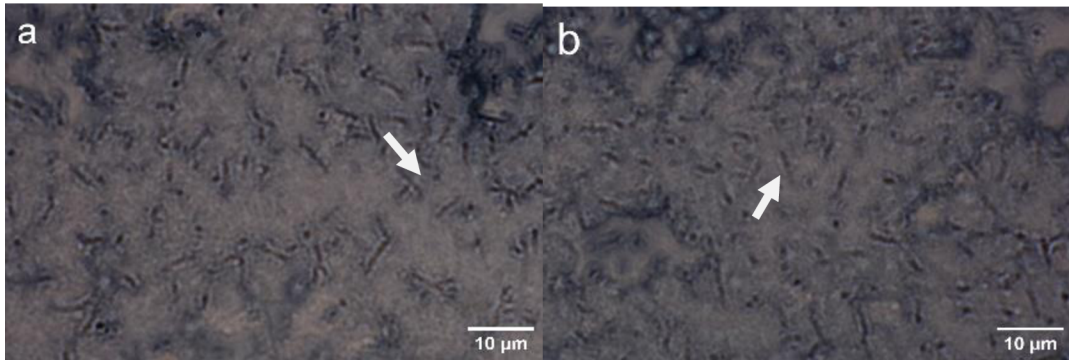violet from crystal violet, or nigrosine, respectively. Anthony's method did not show clear capsules (Figure 13).

Figure 5 Sphingomonas desiccabilis grown in C/N 4 medium differentially stained with nigrosine.
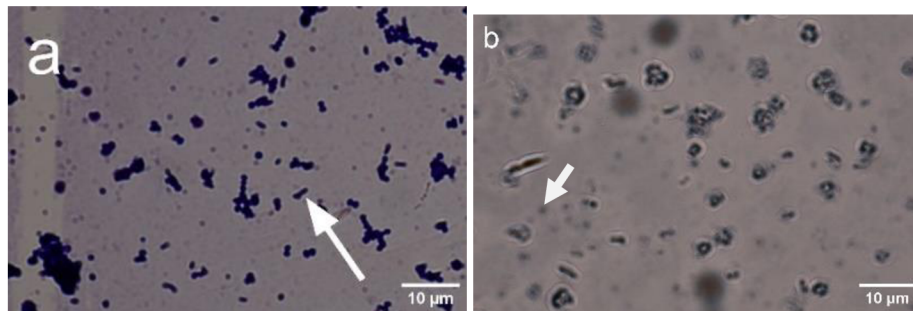

Figure 6 Curtobacterium oceanisedimentum grown in C/N 4 medium differentially stained with Hiss's (a) and nigrosine (b).

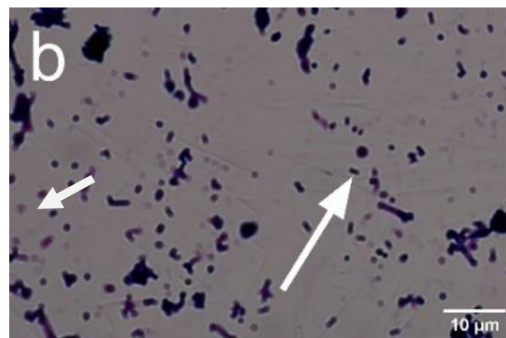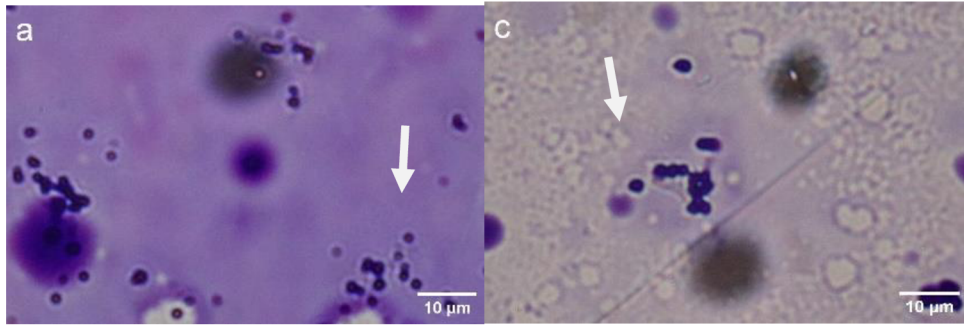*Figure 7 Viridibacillus arvi grown in C/N 4 medium differentially stained with Hiss's method.*

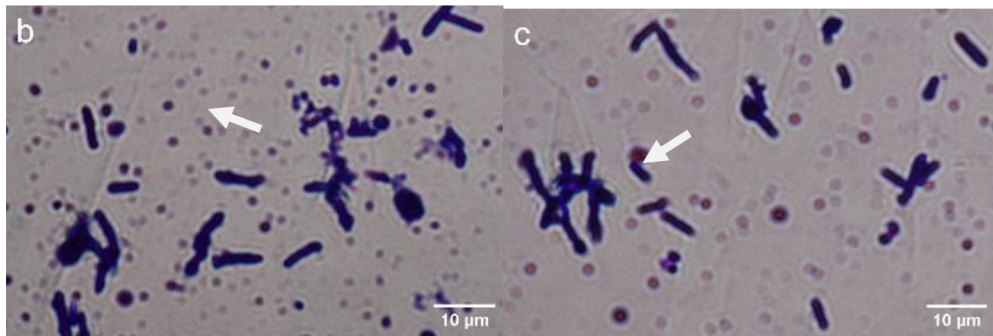*Figure 8 Sphingomonas desiccabilis grown in C/N 8 medium differentially stained with Hiss's method.*

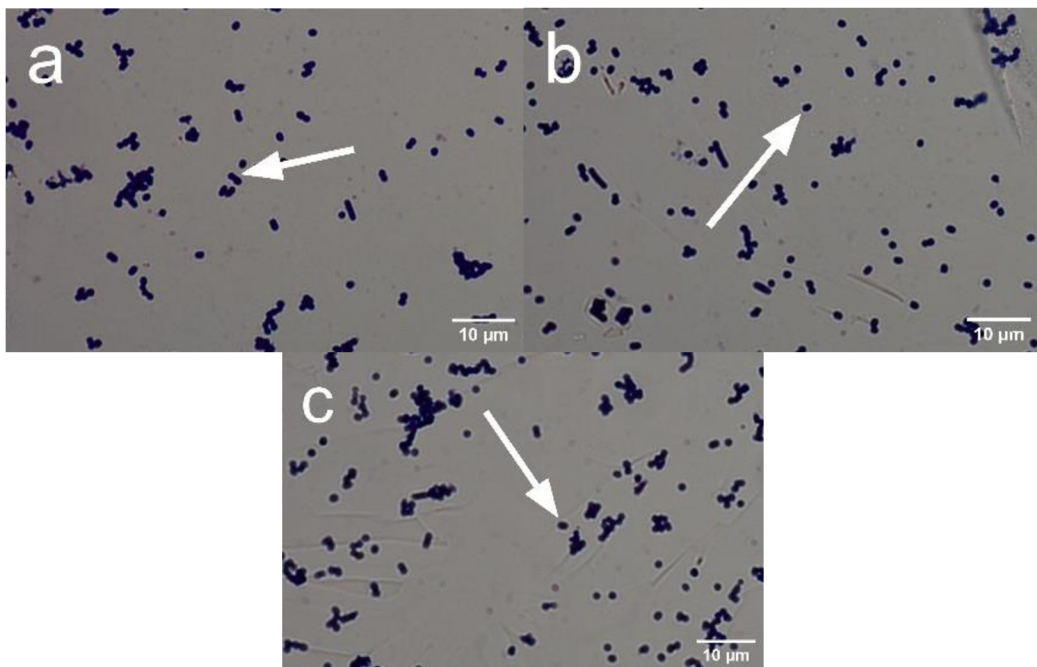*Figure 9 Viridibacillus arvi grown in C/N 8 medium differentially stained with Hiss's method.*

Figure 10 Sphingomonas desiccabilis grown in C/N 16 medium differentially stained with Hiss's method.
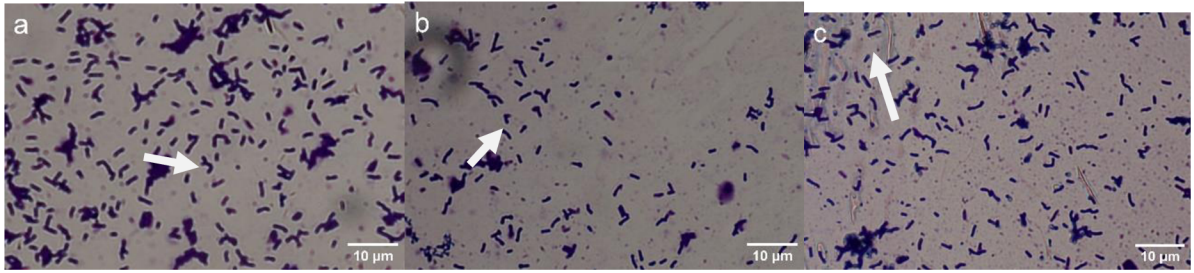
Figure 11 Curtobacterium oceanisedimentum grown in C/N 16 medium differentially stained with Hiss's method.
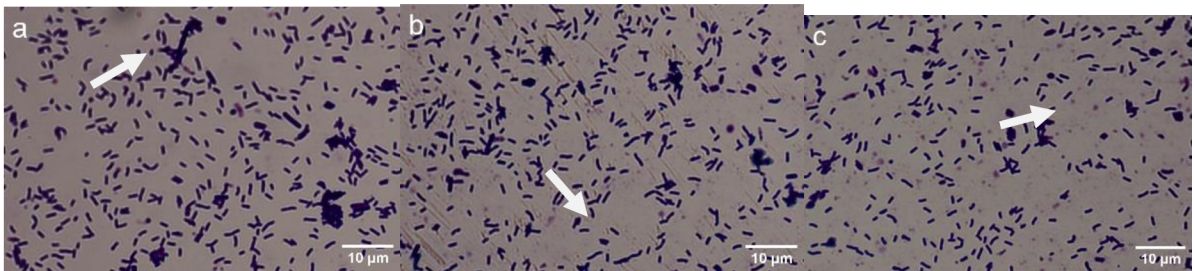
Figure 12 Viridibacillus arvi grown in C/N 16 medium differentially stained with Hiss's method.
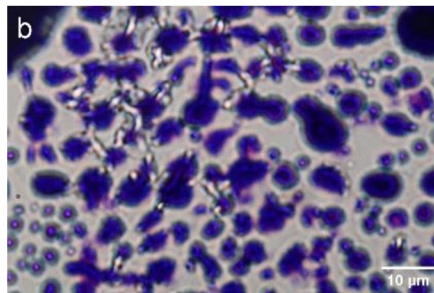
Figure 13 Bacteria differentially stained with Anthony's method.

3.6 CFU

Bacterial solution from the pre-culture preparation was diluted in a dilution series and plated on EPS agar. The count of colony forming units on the incubated Petri dishes numbered on average 368 CFU for *Sphingomonas desiccabilis*, 12 CFU for *Curtobacterium oceanisedimentum,* and 14 CFU *Viridibacillus arvi* for the 10^6-fold dilution. This shows a faster growth for *Sphingomonas desiccabilis* than for *Curtobacterium oceanisedimentum* and *Viridibacillus arvi*. The average CFU for all three bacterial strains increased from C/N 4 (115 CFU) to C/N 8 (170 CFU) and then again decreased to C/N 16 (109 CFU). These

values result in an average CFU of $6.61 \cdot 10^8$ for the C/N 4, $9.20 \cdot 10^8$ for the C / N 8 and $6.04 \cdot 10^8$ for the C/N 16 medium.

The CFU ml^{-1} for *Curtobacterium oceanisedimentum* and *Viridibacillus arvi* changed from $6.67 \cdot 10^6$ and $1.07 \cdot 10^8$, respectively (C/N 4) to $1.77 \cdot 10^8$ and $6.37 \cdot 10^7$, respectively (C/N 8) and further to $4.53 \cdot 10^7$ and $1.06 \cdot 10^6$, respectively (C/N 16) (Figure 12).

The CFU ml^{-1} for *Curtobacterium oceanisedimentum* was highest for C/N 8 ($1{,}77 \cdot 10^8$), whereas for *Viridibacillus arvi* it was almost equal highest for C/N 4 and C/N 16 ($1{,}07 \cdot 10^8$ and $1{,}06 \cdot 10^8$, respectively). The CFU ml^{-1} for *Curtobacterium oceanisedimentum* was lowest for C/N 4 ($6{,}67 \cdot 10^6$), while for *Viridibacillus arvi* it was lowest for C/N 8 ($6{,}37 \cdot 10^7$). The CFU ml^{-1} for *Sphingomonas desiccabilis* (positive control) was higher than for the sample strains, whereas the BOD lacks such a difference between the samples and the positive control. Table 6 displays the summary of the CFU ml^{-1}. In summary, there are only very minor differences in CFU ml^{-1} for both *Sphingomonas desiccabilis* ($p = 0.86$) and *Viridibacillus arvi* ($p = 0.97$), only *Curtobacterium oceanisedimentum* demonstrates a pronounced increase from C/N 4 to C/N 8 ($p = 0.071$) and then a decrease from C/N 8 to C/N 16.
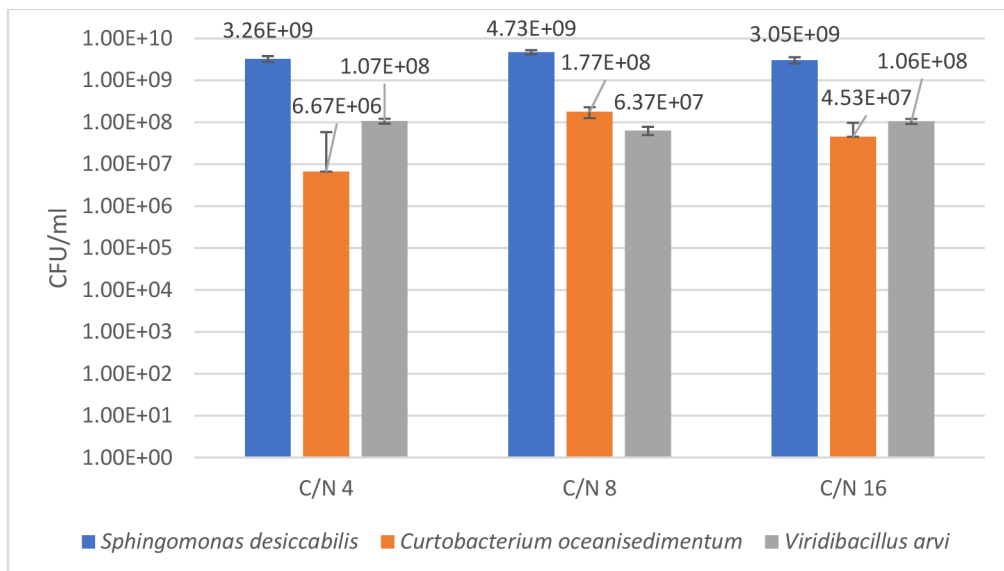

Figure 14 Graphical representation of the CFU ml^{-1} on a logarithmic scale.

4. Discussion

In this experiment, the impact of C/N ratio on EPS production in *Curtobacterium oceanisedimentum* and *Viridibacillus arvi* was investigated. We measured the BOD and CO_2 during incubation in mg l^{-1} and determined the EPS dry weight afterwards, and the CFU ml^{-1}. A decrease in BOD with increasing C/N ratio was found for *Curtobacterium oceanisedimentum* and *Viridibacillus arvi*, respectively. The EPS increased from C/N 4 to C/N 8 for *Curtobacterium oceanisedimentum* and *Viridibacillus arvi*. Both the purity of the bacterial strains and the existence of capsules were confirmed by differential staining and microscopy. Furthermore, only very minor differences in CFU ml^{-1} were found for both *Sphingomonas desiccabilis* and *Viridibacillus arvi,* only *Curtobacterium oceanisedimentum* demonstrates a pronounced increase from C/N 4 to C/N 8 and then a decrease from C/N 8 to C/N 16.

The data show that there is no general trend observable with respect to BOD and CFU. However, the CO_2 of all strains and the BOD of *Curtobacterium oceanisedimentum* seems to decrease with increasing C/N ratio indicating lowered bacterial activity. This relationship was unexpected, as an increase in C substrate could lead to increased bacterial activity, especially, as the easily degradable glucose is used as a carbon source (Almansoory et al., 2020). However, Delbarre-Ladrat et al. (2022) showed that both N and C sources must be increased at the same time. Otherwise, the production yield, which depends on bacterial activity and therefore BOD, might not increase.

The increase in EPS produced by *Curtobacterium oceanisedimentum* and *Viridibacillus arvi* with increasing C/N ratio can be explained by the fact that a higher C/N ratio favours increased EPS production due to a higher amount of available C. *Sphingomonas desiccabilis*, however, did not produce a higher mass in a higher C/N ratio, which could be due to the higher C dependence of this strain, as it is less prone to nutrient shortage than the permafrost strains. The lack of an increase of the EPS at C/N 16 exhibits an unexpected manner, which should be further investigated with the use of higher C/N ratios.

The existence of well-observable capsules around the cells indicated the pronounced production of EPS by all three bacterial strains, while no difference in amount or visibility between the C/N ratio or the bacterial strains could be observed. Hiss's method and nigrosine staining better showed the capsules, while Anthony's method lacks clear visibility of both the bacterial cells and the capsules, which could be due to the too-high concentration of crystal violet concentration. Nigrosine has the highest contrast between the stained cells

and the unstained capsules. Hiss's method has a lower contrast than nigrosine, though still well observable. The washing of the remaining stain seems to have worked best for Hiss's method, enabling a clearer sight on the capsules compared to Anthony's method, which has almost no contrast between the cells and capsules. Both the staining and the washing of the leftover stain did not work well for Anthony's method, as the cells cannot be clearly distinguished from the stain. Despite washing with copper sulphate solution, stain remained on the microscopy slides, making the observation of the capsules more difficult. Furthermore, the area of the capsules for different C/N ratios and different bacterial strains were very similar to each other, so that the aim to quantify the EPS based on the microscopy pictures was not achieved. Therefore, it can be summarized that the staining methods still must be further optimised to enable an image analysis including the quantification of the EPS.

Only *Curtobacterium oceanisedimentum* demonstrates a pronounced increase in CFU ml^{-1} from C/N 4 to C/N 8 but then decreases again from C/N 8 to C/N 16 meaning that this strain is only saturated for growth with easily available C at C/N 8, while the other two strains are already saturated at C/N 4. However, the CFU values were not accurate, as the bacterial solutions did not always mix very well, and the slimy capsules further caused the cells to stick together. The CFU ml^{-1} values tend to be higher than reported in the literature, which is due to the longer incubation time used in this experiment compared to the literature (24 to 48 h as reported by Asaduzzaman et al. (2008) with resulting CFU ml^{-1} values of $5.03 \cdot 10^7$ for C/N 10 and $5.90 \cdot 10^7$ for C/N 15, respectively). The increase of CFU ml^{-1} during a period of decreased BOD might be due to a period of pronounced bacterial growth, during which the production of EPS is decreased. In fact, Farag et al. (2020) showed that a decreased growth rate can lead to increased EPS production, as a higher number of precursors are then available for EPS synthesis.

This study is limited to the three bacterial strains used in this experiment. Furthermore, the results are probably limited to the specific culture conditions used in this experiment. The most important of them is probably the incubation temperature, which greatly exceeds the temperatures found in the permafrost soil. In this experiment, mostly nonoptimal conditions were used, whereas the literature reported higher concentrations of C and N, different sources of N, and much higher shaking speeds (Almansoory et al., 2020; Delbarre-Ladrat et al., 2022; Farag et al., 2020). Therefore, the impact of the C/N ratio could greatly differ for growth conditions different from those used in this study.

5. Conclusion

In this study, the impact of the C/N ratio on EPS production was investigated in *Curtobacterium oceanisedimentum* and *Viridibacillus arvi.* A decrease in BOD with increasing C/N ratio was found for *Curtobacterium oceanisedimentum* and *Viridibacillus arvi*, respectively. The EPS increased with increasing C/N ratio until C/N 8 for *Curtobacterium oceanisedimentum* and *Viridibacillus.* Hiss's method was found to be the best method for differential staining, though all methods must be further improved. Furthermore, *Curtobacterium oceanisedimentum* demonstrates a pronounced increase in CFU ml^{-1} from C/N 4 to C/N 8 and then a decrease from C/N 8 to C/N 16. While investigating the impact of C/N ratio on EPS production, no general trend could be observed. The BOD and, therefore, the potential for EPS production could not be significantly increased by increasing the C/N ratio in the scope of this study. The impact of a higher range of C/N ratios, as well as different culturing conditions, should be further investigated. Furthermore, a direct quantification of the EPS produced should be used in future studies.

6. References

Alami, Y., Achouak, W., Marol, C., and Heulin, T. (2000). Rhizosphere soil aggregation and plant growth promotion of sunflowers by an exopolysaccharide-producing Rhizobium sp strain isolated from sunflower roots. Appl. Environ. Microbiol. 66, 3393–3398. doi: 10.1128/Aem.66.8.3393-3398.2000

Almansoory, A. F., Al-Baldawi, I. A., & Hazaimeh, M. (2020). Optimization of the EPS production of a bacterial floc consortium using different parameters. *Biocatalysis and Agricultural Biotechnology*, *23*, 101466. https://doi.org/https://doi.org/10.1016/j.bcab.2019.101466

Anisimov, O. A., Nelson, F. E., & Pavlov, A. V. (1999). Predicative scenarios of permafrost development under conditions of global change in the XXI century. Earth's Cryology, 3, 15–25.

Asaduzzaman, M., Wahab, M. A., Verdegem, M. C. J., Huque, S., Salam, M. A., & Azim, M. E. (2008). C/N ratio control and substrate addition for periphyton development jointly enhance freshwater prawn Macrobrachium rosenbergii production in ponds. *Aquaculture*, *280*(1), 117–123. https://doi.org/https://doi.org/10.1016/j.aquaculture.2008.04.019

Bayer, M. E. (1990). Visualization of the bacterial polysaccharide capsule. *Bacterial capsules*, 129-157.

Becker, A., Küster, H., Niehaus, K., & Pühler, A. (1995). Extension of the Rhizobium meliloti succinoglycan biosynthesis gene cluster: identification of the exsA gene encoding an ABC transporter protein, and the exsB gene which probably codes for a regulator of succinoglycan biosynthesis. *Molecular and General Genetics MGG*, *249*, 487-497.

Bhattacharjee, A., Thompson, A. M., Schwarz, K. C., Burnet, M. C., Kim, Y. M., Nunez, J. R., Fansler, S. J., Farris, Y., Brislawn, C. J., Metz, T. O., McClure, R. S., Renslow, R. S., Shor, L., Jansson, J. K., Hofmockel, K. S., & Anderton, C. R. (2020). Soil microbial EPS resiliency is influenced by carbon source accessibility. *Soil Biology and Biochemistry*, *151*. https://doi.org/10.1016/j.soilbio.2020.108037

Celik, G.Y., Aslim, B., Beyatli, Y. 2008. Characterization and production of the exopolysaccharide (EPS) from Pseudomonas aeruginosa G1 and *Pseudomonas putida*

G12 strains. Carbohydrate Polymers, 73, 178–182. http://dx.doi.org/10.1016/j.carbpol.2007.11.021

Chai, Y., Cai, P., Gillor, O., Kuramae, E. E., A Costa, O. Y., & Raaijmakers, J. M. (2018). *Microbial Extracellular Polymeric Substances: Ecological Function and Impact on Soil Aggregation*. https://doi.org/10.3389/fmicb.2018.01636

Chawla, P. R., Bajaj, I. B., Survase, S. A., & Singhal, R. S. (2009). Microbial cellulose: fermentative production and applications. Food Technology & Biotechnology, 47(2).

Chenu, C. (1995). "Extracellular polysaccharides: an interface between microorganisms and soil constituents," in Environmental Impact of Soil Component Interactions. Natural and Anthropogenic Organics, eds P. M. Huang, J. Berthelin, J. M. Bollag, W. B. Mcgill, and A. L. Page (Boca Raton, FL: CRC Lewis Publishers).

Chong, B. F., Blank, L. M., Mclaughlin, R., and Nielsen, L. K. (2005). Microbial hyaluronic acid production. Appl. Microbiol. Biotechnol. 66, 341–351. doi: 10.1007/s00253-004-1774-4

Comte, S., Guibaud, G., Baudu, M. Relations between extraction protocols for activated sludge extracellular polymeric substances (EPS) and EPS complexation properties: part I. Comparison of the efficiency of eight EPS extraction methods. Enzyme Microb. Technol., 38 (1–2) (2006), pp. 237-245

Cuthbertson, L., Mainprize, I. L., Naismith, J. H., and Whitfield, C. (2009). Pivotal roles of the outer membrane polysaccharide export and polysaccharide copolymerase protein families in export of extracellular polysaccharides in gram-negative bacteria. Microbiol. Mol. Biol. Rev. 73, 155–177. doi: 10.1128/MMBR.00024-08

Delbarre-Ladrat, C., Sinquin, C., Marchand, L., Bonnetot, S., Zykwinska, A., Verrez-Bagnis, V., & Colliec-Jouault, S. (2022). Influence of the Carbon and Nitrogen Sources on Diabolican Production by the Marine Vibrio diabolicus Strain CNCM I-1629. *Polymers*, *14*(10). https://doi.org/10.3390/polym14101994

Dobinski, W. (2011). Permafrost. *Earth-Science Reviews*, *108*(3–4), 158–169. https://doi.org/10.1016/J.EARSCIREV.2011.06.007

Duguid, J. P. (1951). The demonstration of bacterial capsules and slime. *Journal of Pathology and Bacteriology*, *63*(4), 673-85.

Farag, M. M. S., Moghannem, S. A. M., Shehabeldine, A. M., & Azab, M. S. (2020). Antitumor effect of exopolysaccharide produced by Bacillus mycoides. *Microbial Pathogenesis*, *140*, 103947. https://doi.org/10.1016/J.MICPATH.2019.103947

Finan, T. M., Kunkel, B., De Vos, G. F., and Signer, E. R. (1986). Second symbiotic megaplasmid in Rhizobium meliloti carrying exopolysaccharide and thiamine synthesis genes. J. Bacteriol. 167, 66–72.

Flemming, H.-C., Wingender, J. The biofilm matrix. Nature Reviews Microbiology, 8 (2010), pp. 623-633, 10.1038/nrmicro2415

Flemming, H.-C., Wingende, J., Szewzyk, U., Steinberg, P., Rice, S.A., Kjelleberg, S. Biofilms: an emergent form of bacterial life. Nature Reviews Microbiology, 14 (2016), pp. 563-575, 10.1038/nrmicro.2016.94

Fontaine, S., Barot, S., Barre, P., Bdioui, N., Mary, B., & Rumpel, C. (2007). Stability of organic carbon in deep soil layers controlled by fresh carbon supply. Nature, 450, 277–280. https://doi.org/10.1038/nature06275

Funke, G., Aravena-Roman, M., & Frodl, R. (2005). First description of Curtobacterium spp. isolated from human clinical specimens. *Journal of Clinical Microbiology*, *43*(3), 1032–1036. https://doi.org/10.1128/JCM.43.3.1032-1036.2005

Glucksmann, M. A., Reuber, T. L., & Walker, G. (1993). Genes needed for the modification, polymerization, export, and processing of succinoglycan by Rhizobium meliloti: a model for succinoglycan biosynthesis. Journal of bacteriology, 175(21), 7045-7055.

Gocke, M., Kuzyakov, Y., & Wiesenberg, G. L. B. (2010). Rhizoliths in loess: Evidence for post-sedimentary incorporation of root-derived organic matter in terrestrial sediments as assessed from molecular proxies. *Organic Geochemistry* 41, 1198–1206. https://doi.org/10.1016/j.orggeochem.2010.08.001

Hillel, D. 1982. Introduction to soil Physics. Academic Press Limited, 24-28. Oval Road, London

Hugelius, G.; Strauss, J.; Zubrzycki, S.; Harden, J.D.; Schuur, E.A.G.; Ping, C.L.; Schirrmeister, L.; Grosse, G.; Michaelson, G.J.; Koven, C.D. Estimated stocks of circumpolar permafrost carbon with quantified uncertainty ranges and identified data gaps. Biogeosciences 2014, 11, 6573–6593

Imeson, A. (2010). Food Stabilisers, Thickeners and Gelling Agents.

Islam, S. T., Taylor, V. L., Qi, M., and Lam, J. S. (2010). Membrane topology mapping of the O-antigen flippase (Wzx), polymerase (Wzy), and ligase (WaaL) from Pseudomonas aeruginosa PAO1 reveals novel domain architectures. mBio 1. doi: 10.1128/mBio.00189-10

Jin, H., & Ma, Q. (2021). *Impacts of Permafrost Degradation on Carbon Stocks and Emissions under a Warming Climate: A Review*. https://doi.org/10.3390/atmos12111425

Jurášková, D., Ribeiro, S. C., & Silva, C. C. G. (2022). Exopolysaccharides Produced by Lactic Acid Bacteria: From Biosynthesis to Health-Promoting Properties. In *Foods* (Vol. 11, Issue 2). MDPI. https://doi.org/10.3390/foods11020156

Khvorostyanov, D. V, Ciais, P., Krinner, G., Zimov, S. A., Corradi, Ch., & Guggenberger, G. (2008). Vulnerability of permafrost carbon to global warming. Part II: sensitivity of permafrost carbon stock to global warming. *Tellus B: Chemical and Physical Meteorology*, *60*(2), 265–275. https://doi.org/10.1111/j.1600-0889.2007.00336.x

Konnova, S.A., Brykova, O.S., Sachkova, O.A., Egorenkova, I.V., Ignatov, V.V. 2001. Protective role of the polysaccharide containing capsular components of Azospirillum brasilense. Microbiology, (Moscow, Russ. Ed.), 70, 436–440. http://dx.doi.org/10.1023/a:1010434227671

Kumar, A.S., Mody, K., Jha, B. 2007. Bacterial exopolysaccharides – a perception. Jounal of Basic Microbiology, 47, 103-117. http://dx.doi.org/10.1002/jobm.200610203

Lawrence, D. M., Koven, C. D., Swenson, S. C., Riley, W. J., & Slater, A. G. (2015). Permafrost thaw and resulting soil moisture changes regulate projected high-latitude CO2 and CH4 emissions. Environmental Research Letters, 10, 094011. https://doi.org/10.1088/1748-9326/10/9/094011

Martens, D. A., & Frankenberger, W. T. (1993). Soil saccharide extraction and detection. *Plant and soil*, *149*, 145-147.

Miqueleto, A. P., Dolosic, C. C., Pozzi, E., Foresti, E., & Zaiat, M. (2010). Influence of carbon sources and C/N ratio on EPS production in anaerobic sequencing batch biofilm reactors for wastewater treatment. *Bioresource Technology*, *101*(4), 1324–1330. https://doi.org/10.1016/j.biortech.2009.09.026

Mishra, U., Hugelius, G., Shelef, E., Yang, Y., Strauss, J., Lupachev, A., Harden, J. W., Jastrow, J. D., Ping, C.-L., Riley, W. J., Schuur, E. A. G., Siewert, M., Nave, L. E., Koven, C. D., Fuchs, M., Palmtag, J., Kuhry, P., Treat, C. C., Zubrzycki, S., ... Orr, A. (2021). A P P L I E D E C O L O G Y Spatial heterogeneity and environmental predictors of permafrost region soil organic carbon stocks. In *Sci. Adv* (Vol. 7). https://www.science.org

More, T.T., Yadav, J.S.S., Yan, S., Zyagi, R.R., Surampalli, R.Y. Extracellular polymeric substances of bacteria and their potential environmental applications. Journal of Environmental Management, 144 (2014), pp. 1-25, 10.1016/j.jenvman.2014.05.010

Morona, R., Purins, L., Tocilj, A., Matte, A., and Cygler, M. (2009). Sequence-structure relationships in polysaccharide co-polymerase (PCP) proteins. Trends Biochem. Sci. 34, 78–84. doi: 10.1016/j.tibs.2008.11.001

Morse, S.R., 1990. Water balance in Hemizonia luzulifolia: the role of extracellular polysaccharides. Plant Cell and Environment, 13, 39-48. http://dx.doi.org/10.1111/j.1365-3040.1990.tb01297.x

Mueller, C., Rethemeyer, J., Kao-Kniffin, J., Loeppmann, S., Hinkel, K., & Bockheim, J. (2015). Large amounts of labile organic carbon in permafrost soils of Northern Alaska. Global Change Biology, 21, 2804–2817. https://doi.org/10.1111/gcb.12876

Nandal, K., Anita, R.S., Attar, S.Y., et al., High temperature-induced changes in exopolysaccharides, lipopolysaccharides and protein profile of heat resistant mutants of Rhizobium sp. (Cajanus), Microbiol. Res., 2005, vol. 160, pp. 367–373.

Natali, S.M.; Watts, J.D.; Rogers, B.M.; Potter, S.; Ludwig, S.M.; Selbmann, A.K.; Sullivan, P.F.; Abbott, B.W.; Arndt, K.A.; Birch, L.; et al. Large loss of CO2 in winter observed across the northern permafrost region. Nat. Clim. Chang. 2019, 9, 852–857.

Nelson, F., Anisimov, O., & Shiklomanov, N. (2001). Subsidence risk from thawing permafrost. Nature, 410, 889–890. https://doi.org/10.1038/35073746

Nichols, M.C.A., Garson, S., Bowman, J.P., et al., Production of exopolysaccharides by Antarctic marine bacterial isolates, J. Appl. Microbiol., 2004, vol. 96, pp. 1057–1066.

Nichols, C.M., Guezennec, J., J. Bowman, J.P. 2005. Bacterial exopolysaccharides from extreme marine environments with special consideration of the Southern Ocean, Sea

Ice, and Deep-Sea hydrothermal vents: a review. Marine Biotechnology, 7, pp 253–271. http://dx.doi.org/10.1007/s10126-004-5118-2

Oleksy, M., & Klewicka, E. (2017). Capsular Polysaccharides of Lactobacillus spp.: Theoretical and Practical Aspects of Simple Visualization Methods. *Probiotics and Antimicrobial Proteins*, *9*(4), 425–434. https://doi.org/10.1007/s12602-017-9295-5

Prater, I., Zubrzycki, S., Buegger, F., Zoor-Fuellgraff, L. C., Angst, G., Dannenmann, M., & Müller, C. W. (2020). From fibrous plant residues to mineral-associated organic carbon - The fate of organic matter in Arctic permafrost soils. Biogeosciences, 17, 3367–3383. https://doi.org/10.5194/bg-17-3367-2020

Read, R.R., Costerton, J.W. 1987. Purification and characterization of adhesive exopolysaccharides from Pseudomonas putida and *Pseudomonas fuorescens*. Canadian Journal of Microbiology, 33, 1080-1090. http://dx.doi.org/10.1139/m87-189

Rehm, B. (2009). Microbial production of biopolymers and polymer precursors.

Rehm, B.H.A. 2010. Bacterial polymers: biosynthesis, modifications and applications. Nature Reviews Microbiology, 8, 578-592. http://dx.doi.org/10.1038/nrmicro2354

Rehm, B., and Valla, S. (1997). Bacterial alginates: biosynthesis and applications. Appl. Microbiol. Biotechnol. 48, 281 –288. doi: 10.1007/s002530051051

Ro, V. O., Ya, S. T., Ov, N., & Ov, M. (2008). Vulnerability of permafrost carbon to global warming. Part II: sensitivity of permafrost carbon stock to global warming. *The Authors Journal Compilation C*, *60*, 265–275. https://doi.org/10.1111/j.1600-0889.2007.00336.x

Ross, P., Weinhouse, H., Aloni, Y., Michaeli, D., Weinberger-Ohana, P., Mayer, R., Braun, S., De Vroom, E., Van der Marel, G. A., Van Boom, J. H., Benziman, M. (1987). Regulation of cellulose synthesis in Acetobacter xylinum by cyclic diguanylic acid. Nature, 325(6101), 279-281.

Rühmann, B., Schmid, J., & Sieber, V. (2015). High throughput exopolysaccharide screening platform: From strain cultivation to monosaccharide composition and carbohydrate fingerprinting in one day. *Carbohydrate Polymers*, *122*, 212–220. https://doi.org/https://doi.org/10.1016/j.carbpol.2014.12.021

Sandhya, V., & Ali, Sk. Z. (2015). The production of exopolysaccharide by Pseudomonas putida GAP-P45 under various abiotic stress conditions and its role in soil aggregation. *Microbiology*, *84*(4), 512–519. https://doi.org/10.1134/S0026261715040153

Schmid, J., Sieber, V., & Rehm, B. (2015). Bacterial exopolysaccharides: biosynthesis pathways and engineering strategies. *Frontiers in Microbiology*, *6*. https://doi.org/10.3389/fmicb.2015.00496

Schuur, E.A.G.; McGuire, A.D.; Schädel, C.; Grosse, G.; Harden, J.W.; Hayes, D.J.; Hugelius, G.; Koven, C.D.; Kuhry, P.; Lawrence, D.M.; et al. Climate change and the permafrost carbon feedback. Nature, 2015, 520, 171–179

Schuur, E. A. G., Abbott, B. W., Commane, R., Ernakovich, J., Euskirchen, E., Hugelius, G., Grosse, G., Jones, M., Koven, C., Leshyk, V., Lawrence, D., Loranty, M. M., Mauritz, M., Olefeldt, D., Natali, S., Rodenhizer, H., Salmon, V., Schädel, C., Strauss, J., Treat, C., Turetsky, M. (2022). Permafrost and Climate Change: Carbon Cycle Feedbacks From the Warming Arctic. *Annual Review of Environment and Resources*, *47*(1), 343–371. https://doi.org/10.1146/annurev-environ-012220-011847

Schuur, E.A.G.; Bockheim, J.; Canadell, J.G.; Euskirchen, E.; Field, C.B.; Goryachkin, S.V.; Hagemann, S.; Kuhry, P.; Lafleur, P.M.; Lee, H.; et al. Vulnerability of permafrost carbon to climate change: Implications for the global carbon cycle. Bioscience 2008, 58, 701–714.

Schuur, E. A. G., & Mack, M. C. (2018). Ecological response to permafrost thaw and consequences for local and global ecosystem services. Annual Review of Ecology, Evolution and Systematics, 49, 279–301. https://doi.org/10.1146/annurev-ecolsys-121415-032349

Sheng, G. P., Yu, H. Q., & Li, X. Y. (2010). Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems: A review. *Biotechnology Advances*, *28*(6), 882–894. https://doi.org/10.1016/J.BIOTECHADV.2010.08.001

Smith, A. P., Marín-Spiotta, E., De Graaff, M. A., & Balser, T. C. (2014). Microbial community structure varies across soil organic matter aggregate pools during tropical land cover change. *Soil Biology and Biochemistry*, *77*, 292-303. 10.1016/j.soilbio.2014.05.030

St Jaques, J.-M., & Sauchyn, D. (2009). Increasing winter baseflow and mean annual streamflow from possible permafrost thawing in the Northwest Territories, Canada. Geophysical Research Letters, 36, L01401. https://doi.org/10.1029/2008GL035822

Sutherland, I. W. Biofilm exopolysaccharides: a strong and sticky framework. Microbiology-SGM, 147 (2001), pp. 3-9

Tang, J., Mo, Y., Zhang, J., and Zhang, R. (2011). Influence of biological aggregating agents associated with microbial population on soil aggregate stability. Appl. Soil Ecol. 47, 153–159. doi: 10.1016/j.apsoil.2011.01.001

Tisdall, J. M., & OADES, J. M. (1982). Organic matter and water-stable aggregates in soils. *Journal of soil science*, *33*(2), 141-163.

Vardharajula, S., and Ali, S. Z. (2015). The production of exopolysaccharide by Pseudomonas putida GAP-P45 under various abiotic stress conditions and its role in soil aggregation. Microbiology 84, 512–519. doi: 10.1134/s0026261715040153

Vardharajula, S., & Sk Z, A. (2014). Exopolysaccharide production by drought tolerant *Bacillus spp.* And effect on soil aggregation under drought stress. *Journal of Microbiology, Biotechnology and Food Sciences*, *4*(1), 51–57. https://doi.org/10.15414/jmbfs.2014.4.1.51-57

Vicente-Garcia, V., Rios-Leal, E., Calderon-Dominguez, G., Canizares-Villanueva, R.O., and Olvera-Ramirez, R., Detection, isolation and characterization of exopolysaccharide produced by a strain of Phormidium 94a isolated from an arid zone of Mexico, Biotechnol. Bioeng., 2004, vol. 85, pp. 306–310.

Wang, X., Sharp, C. E., Jones, G. M., Grasby, S. E., Brady, A. L., Dunfield, P. F., et al. (2015). Stable-isotope probing identifies uncultured planctomycetes as primary degraders of a complex heteropolysaccharide in soil. Appl. Environ. Microbiol. 81, 4607–4615. doi: 10.1128/aem.00055-15

Whitney, J. C., and Howell, P. L. (2013). Synthase-dependent exopolysaccharide secretion in Gram-negative bacteria. Trends Microbiol. 21, 63–72. doi: 10.1016/j.tim.2012.10.001

Wiesmeier, M., Urbanski, L., Hobley, E., Lang, B., von Lützow, M., Marin-Spiotta, E, Van Wesemael, B., Rabot, E., Ließ, M., Garcia-Franco, N., Wollschläger, U., Vogel, H.J., Kögel-Knabner, I. (2019). Soil organic carbon storage as a key function of soils-A

review of drivers and indicators at various scales. *Geoderma*, *333*, 149-162. 10.1016/j.geoderma.2018.07.026

Wild, B., Gentsch, N., Čapek, P., Diáková, K., Alves, R. J. E., Bárta, J., et al. (2016). Plant-derived compounds stimulate the decomposition of organic matter in arctic permafrost soils. Scientific Reports, 6, 25607. https://doi.org/10.1038/srep25607

Willis, L. M., Stupak, J., Richards, M. R., Lowary, T. L., Li, J., and Whitfield, C. (2013). Conserved glycolipid termini in capsular polysaccharides synthesized by ATP-binding cassette transporter-dependent pathways in Gram-negative pathogens. Proc. Natl. Acad. Sci. U.S.A. 110, 7868–7873. doi: 10.1073/pnas.1222317110

Willis, L. M., and Whitfield, C. (2013). Structure, biosynthesis, and function of bacterial capsular polysaccharides synthesized by ABC transporter-dependent pathways. Carbohydr. Res. 378, 35–44. doi: 10.1016/j.carres.2013.05.007

Wingender, J., Neu, T. R., and Flemming, H.-C. (1999b). "What are bacterial extracellular polymeric substances?" in Microbial Extracellular Polymeric Substances: Characterization, Structure and Function, eds J. Wingender, T. R. Neu, and H.-C. Flemming (Berlin: Springer), 1–19.

Xu, Y., Zhao, J.-Y., Geng, Y., Yuan, H.-R., Li, L.-L., Wen, M.-L., Li, M.-G., & Tang, S.-K. (2021). Viridibacillus soli sp. nov., isolated from forest soil in Ailaoshan National Nature Reserve. *Archives of Microbiology*, *204*(1), 60. https://doi.org/10.1007/s00203-021-02676-x

Zhang, X., & Bishop, P. L. (2003). Biodegradability of biofilm extracellular polymeric substances. *Chemosphere*, *50*(1), 63-69.

Zimov, S. A., Davydov, S. P., Zimova, G. M., Davydova, A. I., Schuur, E. A. G., Dutta, K., & Chapin Iii, F. S. (2006). *Permafrost carbon: Stock and decomposability of a globally significant carbon pool*. https://doi.org/10.1029/2006GL027484

7. Appendices

Table A1 Measured headspace pressure and calculated BOD of the incubation experiment.

Experiment number	Sample name	Headspace pressure / hPa	BOD / mg l⁻¹
1 (C/N = 4)	Control	-16	84.02916
	Sphingomonas desiccabilis	-63	332.6154
	Curtobacterium oceanisedimentum	-66	346.6203
	Viridibacillus arvi		442.9037
2 (C/N = 8)	Control	-21	115.5401
	Sphingomonas desiccabilis	-51	271.3442
	Curtobacterium oceanisedimentum	-52	274.8454
	Viridibacillus arvi	-100	528.6835
3 (C/N = 16)	*Sphingomonas desiccabilis*	-46	7.604202
	Curtobacterium oceanisedimentum	-46	7.549495
	Viridibacillus arvi	-82	13.29368

Table A2 CFU on Petri dishes incubated with differently diluted bacterial solutions.

Bacterial strain	Dilution Factor	CFU (C/N 4)	CFU (C/N 8)	CFU (C/N 16)
Sphingomonas desiccabilis-1	10^4	500++	500++	500++
	10^5	500+	500+	500+
	10^6	236	484	276
Sphingomonas desiccabilis-2	10^4	500++	500++	
	10^5	500+	500+	
	10^6	425	512	154
Sphingomonas desiccabilis-3	10^4	500++	500++	500++
	10^5	500+	500+	500+
	10^6	318	423	485
Curtobacterium oceanisedimentum-1	10^4	35	140	25
	10^5	6	90	4
	10^6	0	40	1
Curtobacterium oceanisedimentum-2	10^4	43	49	44
	10^5	9	26	8
	10^6	0	23	1
Curtobacterium oceanisedimentum-3	10^4	122	75	78
	10^5	5	29	29
	10^6	1	20	22
Viridibacillus arvi-1	10^4	524	349	500+
	10^5	89	18	190
	10^6	17	3	10
Viridibacillus arvi-2	10^4	644	392	456
	10^5	112	52	84
	10^6	27	7	21
Viridibacillus arvi-3	10^4	561	411	333
	10^5	106	78	63
	10^6	10	21	12