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Structure and enzymatic degradation of the polysaccharide secreted by *Nostoc commune*

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► **To cite this version:**

Sophie Drouillard, Laurent Poulet, Eric Marechal, Alberto Amato, Laurine Buon, et al.. Structure and enzymatic degradation of the polysaccharide secreted by *Nostoc commune*. *Carbohydrate Research*, 2022, 515, pp.108544. 10.1016/j.carres.2022.108544 . hal-03630667

HAL Id: hal-03630667

<https://hal.science/hal-03630667v1>

Submitted on 5 Apr 2022

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26 1. Introduction

27 The *Nostoc* genus groups very diverse species of filamentous cyanobacteria that form microscopic or
28 macroscopic colonies embedded in a gelatinous matrix. The colonies may adopt various morphologies:
29 sheet-like (*N. commune*), hair-like (*N. flagelliform*) or spheric morphology (*N. pruniform*, *N. sphaeroides*)
30 as a function of the species and their growth conditions. *Nostoc sp.* have been collected and used for food
31 and medicine for centuries in many places in the world. Currently, *Nostoc flagelliform* (“facai”) in China
32 and *Nostoc pruniform* (“lullucha”) in South America are nowadays probably the most used for their
33 nutritive properties as diet complement (Dodds & Gudder, 1995; Gao, 1998; Qui, Liu J., Liu Z. & Liu S.,
34 2002; Sand-Jensens, 2014). These microorganisms may be found in various and extreme, terrestrial and
35 aquatic environments. They tolerate the freezing temperature of Antarctic (e.g. *Nostoc commune*) (Davey,
36 1989; Novis et al., 2007), high temperature of desert (e.g. *Nostoc flagelliform*; Gao, 1998, Hu, Liu,
37 Paulsen, Petersen & Klaveness, 2003) and sun radiation found in the altitude of Andes (Fleming &
38 Prufert-Bebout, 2010). The exceptional capacity of *Nostoc spp.* to live in poor habitat and to adapt to
39 extreme environments is explained by their metabolisms and their capacity to resist to desiccation (Dodds
40 & Gudder, 1995; Sand-Jensen, 2014). As other cyanobacteria, *Nostoc spp.* can fix carbon by
41 photosynthesis and they developed strategies to incorporate atmospheric nitrogen. To face desiccation,
42 cyanobacterial cells differentiate into akinete cells (resting-state cells of cyanobacteria) which germinate
43 when conditions become more favorable (Perez, Forchhammer, Salerno & and Maldener, 2016).

44 The gelatinous matrix embedding the cyanobacterial colonies is mainly composed of a high molecular
45 weight polysaccharide forming a highly viscous solution. The physicochemical properties of the
46 polysaccharide and its capacity to retain water is correlated to the structural properties of the
47 macromolecule. Composition analyses of the secreted polysaccharide conducted on numerous species and
48 strains of *Nostoc* revealed that some neutral monosaccharide residues (glucose: Glc; galactose: Gal;
49 xylose: Xyl) and uronic residues (glucuronic acid:GlcA) are very often present. Other pentose residues
50 such as arabinose (Ara) and ribose (Rib), and organic groups (lactate, pyruvate) also found in the
51 composition increase the complexity of the polysaccharides (Huang, Liu, Paulsen & Klaveness, 1998;
52 Brüll et al., 2000; Pereira et al., 2009).

53 The first structure of the polysaccharides secreted by two strains of *Nostoc commune* collected in Asia
54 were determined by Helm and co-workers (2000) and Brüll and co-workers (2000). The structures showed
55 a similar tetrasaccharide repeating unit of the main chain made of [\rightarrow 4) β -D-Glc (\rightarrow 4) α -D-Gal (\rightarrow 4) β -
56 D-Glc (\rightarrow 4) Xyl (\rightarrow] carrying an uronic residue (glucuronic acid: GlcA, or nosturonic acid: pyruvated-
57 GlcA) and a deoxy residue (Ara, Rib). The structure of the polysaccharides, and more especially the
58 decoration of the main chain, was found to change with growth conditions (Brüll et al., 2000).

59 The exact biological role of the matrix polysaccharide is not well understood but it probably
60 participate in the survival of *Nostoc spp.* by retaining water in dry environment and by participating in the
61 rewetting of the akinete cells prior they germinate when environmental conditions are favorable.
62 Assuming that the biological role in the survival of *Nostoc* cells is driven by the structural features of the
63 macromolecules, we hypothesized that their chemical characteristics were probably conserved in the
64 *Nostoc commune* species and may be shared with other species of the genus *Nostoc*. In this context, we
65 have analyzed the polysaccharide structure of a strain of *Nostoc commune* collected in Europe. With the
66 help an enzyme catalyzing the degradation of the *Nostoc commune* polysaccharides, recently identified
67 through the systematic screening of distantly related carbohydrate active enzymes (Helbert et al. 2019),
68 we have produced a series of oligosaccharides helping in the resolution of the chemical structure of the
69 polysaccharide.

70 **2. Material and methods**

71 2.1. DNA extraction

72 Frozen sheet-like *Nostoc commune* were grinded to fine powder in liquid nitrogen. The sample was
73 suspended in 500µl of lysis buffer (250 mM Tris pH 8.2, 100 mM EDTA, 2% SDS, 100 mM NaCl) and
74 was warmed for 30 minutes at 90°C. During this step, the suspension was vortexed for 30 seconds every
75 10 minutes. Then, 500 µL of phenol:chloroform:isoamyl alcohol (25:24:1) were added to the sample and
76 mixed vigorously to obtain a homogeneous suspension (at least 3 minutes with vortex). In order to
77 separate the cell debris and the aqueous solution containing the nucleic acids, the samples were
78 centrifuged 10 minutes at 17,000 × g at 4°C. The upper phase was transferred to a new tube and 300 µL of
79 chloroform:isoamyl alcohol (24:1) were added. The sample was vigorously mixed by inverting the tubes
80 followed by centrifugation for 10 min at 13,000 × g at 4°C aiming at separating the two phases. The upper
81 aqueous phase was transferred in a new tube and 30µL of 3M Na-acetate and 1.5 volumes (750 µL) of
82 absolute ethanol were added to precipitate the DNA. Precipitation was conducted for at least 20 minutes at
83 -20°C. DNA salts were pelleted by centrifugation (10 min. at 13,000 × g at 4°C). The supernatant was
84 discarded and the pellet was washed twice with 500 µL of 70% ethanol. Washing steps were done by
85 adding ethanol to the pellet and centrifuging 5 minutes at 13,000 × g at 4°C. After the second ethanol
86 washing, the supernatant was carefully removed without disturbing the pellet and it was air dried for 30-60
87 minutes. The pellet was then resuspended in TE buffer. Purity and quantity were controlled with a
88 NanoDrop spectrophotometer.

89 2.2. PCR amplification and sequencing

90 100 ng of the purified DNA was used as template for PCR amplification. CYA359F and
91 CYA781R (Boutte, Grubisic, Balthasart & Wilmotte, 2006) primer pair was used to amplify a 427nt

92 fragment of the 16S rRNA gene. The PCR reactions were performed in 25 μ L total volume, containing 5
93 μ l 5X Phusion HF buffer, 200 μ M (final concentration) dNTPs, 0.5 μ M of each primer, 0.5 units of
94 BioLabs Phusion® High-Fidelity DNA Polymerase (M0530). A Biorad T100™ Thermal Cycler was used
95 for PCR reactions with the following cycles: a desaturation step at 98°C for 30 seconds, a gradient from
96 45 to 60 °C for the annealing (5 temperatures) and an elongation of 15 seconds at 72°C. The PCR products
97 were loaded on a 0.8% agarose TAE gel electrophoresis working for 30 minutes at 100V. The DNA
98 fragment was visualized on a UV/white light transilluminator. All the tested annealing temperatures
99 produced an amplification with a clear band at the expected size. Because no band of no specific
100 amplification was observed on the gel, PCR products were purified using a Macherey-Nagel NucleoSpin®
101 Gel and PCR Clean-up kit following the manufacturer's instructions. Purified PCR products were sent out
102 to Eurofins Genomics for sequencing in both 5' and 3' directions using the amplification primers.
103 Sequences were visualized and assembled in BioEdit Sequence Alignment Editor (Hall, 1999) and blasted
104 against GenBank. All the top hit sequences were downloaded and further analyzed.

105 2.3. Polysaccharide extraction and purification

106 Small pieces of *Nostoc commune* were suspended in distilled water at 100°C for 1h. After
107 removing the water and rinsing twice the *N. commune* fragments with fresh water, the sample was treated
108 at 70°C for 6 h by a bleaching solution which consisted of 1 volume of 1.7% aqueous NaClO₂ and 1
109 volume of acetate buffer (pH 4.9), completed with 3 volumes of distilled water. The sample was washed
110 with distilled water by repeated centrifugation and finally resuspended in 3% (w/v) KOH solution
111 overnight at room temperature. The insoluble fraction was discarded by centrifugation. The
112 polysaccharide was precipitated by adding an equal volume of ethanol to the supernatant. The pellet was
113 washed by repeated centrifugation with water/ethanol solution (1/1, v/v). The polysaccharide was finally
114 suspended in water and lyophilized.

115 2.4. Monosaccharide analysis

116 The molar ratio of the monosaccharides was determined according to (Kamerling, Gerwig,
117 Vliegthart & Clamp, 1975) modified by (Montreuil et al., 1986). The EPS was hydrolysed with 3 M
118 MeOH/HCl at 110°C for 4 h, followed by re-N-acetylation with Ac₂O overnight at room temperature. The
119 methyl glycosides were converted to their corresponding trimethylsilyl derivatives. Separation and
120 quantification of the per-*O*-trimethylsilyl methyl glycosides were performed using gas-liquid
121 chromatography (GLC) on an Agilent system equipped with a HP-5 ms capillary column (Agilent 0.25
122 mm x 30 m). The trimethylsilyl derivatives were analysed using the following temperature program:
123 120°C→180°C at 3°C/min, 180°C→200°C at 2°C/min, 200°C for 5 min.

124 2.5. Methylation analysis

125 Glycosyl linkage positions were determined as described in (Hakomori, 1964). Hydroxyl groups were
126 methylated using the lithium dimethylsulfonyl as the anion and methyl iodide in Me₂SO (Blakeney and
127 Stone, 1985; Kvernheim, 1987). After two methylation steps, the permethylated products was carboxyl-
128 reduced by LiBD₄ in absolute ethanol for 2h at 80°C (Linnerborg and al, 1997). Excess of borodeuteride
129 was decomposed with 10% acetic acid. After evaporation, the permethylated and carboxyl-reduced
130 products were hydrolysed in 2M trifluoroacetic acid (TFA) for 2h at 120°C, then reduced with NaBD₄ in
131 an NH₄OH solution for 30min at 80°C, and finally acetylated with 200µL of 1-methyl imidazole and 2mL
132 acetic anhydride for 10min at room temperature. GC-mass spectrometry (MS) was performed on an
133 Agilent instrument fitted with a Supelco SP2380 capillary column (0.25 mm× 30 m). The temperature
134 program was 150 °C for 2 min, 150 °C → 240 °C at 3 °C/min, 240 °C for 5 min. Ionisation was carried
135 out in electron impact mode (EI, 70 eV). All analyses were conducted in triplicate.

136 2.6. Preparation of the *Nostoc* polysaccharide hydrolase

137 *Nostoc* polysaccharide hydrolase was overexpressed and purified by affinity chromatography as
138 previously reported in Helbert and co-workers (2019). Briefly, the gene encoding the enzymes was cloned
139 in pHTP1 (NZYtech, Portugal) vector harboring a His₆-tag at the N-terminal position. The enzyme was
140 overexpressed in *E. coli* BL21(DE3) pLysS strain grown in NZY auto-induction LB media (NZYtech,
141 Portugal). After a freeze-thaw cycle, the protein was purified by affinity using a nickel agarose affinity
142 resin (Ni-NTA resin, Qiagen) loaded on poly-prep® chromatography columns (Bio-Rad 731-1550). The
143 His₆-tagged recombinant enzyme was eluted with increasing amounts of imidazole (20 mM, 50 mM, 150
144 mM, 300 mM and 500 mM). The purity of the fractions was estimated by 10% SDS-PAGE analysis.

145 2.7. Enzymatic degradation and purification of oligosaccharides

146 Enzymatic degradations were carried out by incubating 10 mL of purified polysaccharides (1%
147 w/v in 20 mM Tris-HCl pH 7.5, 20 mM NaCl) with 500 µL of purified enzyme (0.60µg.µL⁻¹) at 25°C.
148 End-products were purified on a semi-preparative size-exclusion chromatography system which consisted
149 of a Knauer pump (pump model 100), a HW40 Toyopearl column (120 x 16 mm; Tosoh Corporation), a
150 refractive index detector (Iota 2, Precision Instruments) and a fraction collector (Foxy R, Teledyne Isco 1)
151 mounted in series. Elution was conducted at a flow rate of 0.4 mL/min at room temperature using 100 mM
152 (NH₄)₂CO₃ as the eluent. The fractions containing the oligosaccharides were collected and freeze-dried.

153 2.8. NMR

154 Carbon-13 and proton NMR spectra were recorded with a Bruker Avance 400 spectrometer operating
155 at a frequency of 100.618 MHz for ¹³C and 400.13 MHz for ¹H. Samples were solubilized in D₂O at a
156 temperature of 293 K for the oligosaccharides and 353 K for the polysaccharide. Residual signal of the

157 solvent was used as internal standard: HOD at 4.85 ppm at 293 K and 4.25 at 353 K. ¹³C spectra were
 158 recorded using 90° pulses, 20,000 Hz spectral width, 65,536 data points, 1.638 s acquisition time, 1 s
 159 relaxation delay and between 8192 and 16,834 scans. Proton spectra were recorded with a 4006 Hz
 160 spectral width, 32,768 data points, 4.089 s acquisition times, 0.1 s relaxation delays and 16 scans. The ¹H
 161 and ¹³C-NMR assignments were based on ¹H-¹H homonuclear and ¹H-¹³C heteronuclear correlation
 162 experiments (correlation spectroscopy, COSY; heteronuclear multiple-bond correlation, HMBC;
 163 heteronuclear single quantum correlation, HSQC). They were performed with a 4006 Hz spectral width,
 164 2048 data points, 0.255 s acquisition time, 1 s relaxation delay; 32 to 512 scans were accumulated.

165 3. Results and discussion

166 3.1 Identification of the *Nostoc commune* strain

167 The *Nostoc commune* strain was collected at Saint Martin d'Uriage (France, 45°09'53.7"N
 168 5°51'18.9"E). The macroscopic colonies have dark green gelatinous sheet-like morphology. After
 169 extraction, 16S RNA was sequenced and compared with *Nostoc* 16S RNA sequences available in
 170 Genbank. The sequence showed >98% identity with *Nostoc commune* strains isolated in Europe
 171 (AB113665.1, France; AY577536.1, Czech Republic, strain EV1-KK1; KY283050.1, Russia, strain
 172 ACSSI 030), Asia (AB933330.2, Japan, strain KU006; EU178142.1, China, strain AHNG0605;
 173 KY129708.1, India, strain BHU81) and North America (HQ877826.1, Mexico, strain 257-16). The 16S
 174 RNA analyses demonstrated that the cyanobacterial colonies collected share the same molecular
 175 characteristic of *Nostoc commune* strains.

176 **Table 1.** Methylation analysis of the matrix polysaccharide secreted by *Nostoc commune*. The percentage
 177 of the main linkages observed were estimated by measuring the surface area of the signal.

Residue	Partially Methylated Alditol Acetate	Deduced Linkage	Peak surface area (%)
Glucose	1,4,5-Tri-O-acetyl-1-deuterio-2,3,6,-tri-O-methyl-D-glucitol	→4)-Glc p -(1→	22.7
Glucose	1,4,5,6-Tetra-O-acetyl-1-deuterio-2,3,-di-O-methyl-D-glucitol	→4,6)-Glc p -(1→	24.1
Xylose	1,3,4,5-Tetra-O-acetyl-1-deuterio-2-O-methyl-D-xylitol	→3,4)-Xyl p -(1→	7.7
Xylose	1,4,5-Tri-O-acetyl-1-deuterio-2,3 di-O-methyl-D-xylitol	→4)-Xyl p -(1→	8.8
Xylose	1,4-Di-O-acetyl-1-deuterio-2,3,5-tri-O-methyl-D-xylitol	Xyl f -(1→	1.8
Galactose	1,4,5-Tri-O-acetyl-1-deuterio-2,3,6-tri-O-methyl-D-galactitol	→4)-Gal p -(1→	18.1
Glucuronic acid	1,5,6-Tri-O-acetyl-1,6,6-trideuterio-2,3,4-tri-O-methyl-D-glucitol	GlcA p -(1→	1.5

180 3.2 Characterization of the matrix polysaccharide of *Nostoc commune*

181 Composition analysis using gas chromatography, after the complete hydrolysis of the
 182 polysaccharide and trimethylsilyl derivatization of the products, revealed glucose (Glc), galactose (Gal),
 183 xylose (Xyl) and glucuronic acid (GlcA) (% molar ratio Glc/Gal/Xyl/GlcA 1/0.48/0.90/0.14) (Figure S1).

184 Traces of mannose (% molar ratio Glc/Man 1/0.08) were also detected in the purified polysaccharide.
 185 Methylation analyses (Table 1) revealed that the Glcp residues is mainly involved in 1,4- and 1,4,6-
 186 linkages; the Galp residue participate in 1,4 linkages and the Xylp residue is bound by the position 1, 3
 187 and 4. Terminal GlcA was also observed suggesting its branching on the main chain. In addition, we
 188 detected a derivative presenting the same elution time than the 1,4-di-O-acetyl-1-deuterio-2,3,5-tri-O-
 189 methyl-D-xylitol (Figure S2A) which is present in low amount in the xylose standard (Figure S2B). The
 190 fragmentation products of the derivatives were compared (Figure S3) and validated the occurrence of a
 191 terminal xylofuranoside (T-Xylf) involved in a side chain.

192 Carbon and proton NMR recorded on the polymer revealed the occurrence of six different
 193 residues as illustrated by the heteronuclear $^1\text{H}/^{13}\text{C}$ chemical shift correlation (HSQC) presented in Figure
 194 1. The proton correlation systems observed in the homonuclear $^1\text{H}/^1\text{H}$ chemical shift correlation (COSY,
 195 TOCSY), the determination of the chemical shifts and coupling constants (Table 2) confirmed the
 196 occurrence of glucose, galactose, glucuronic acid and xylose residues expected from chemical analyses.

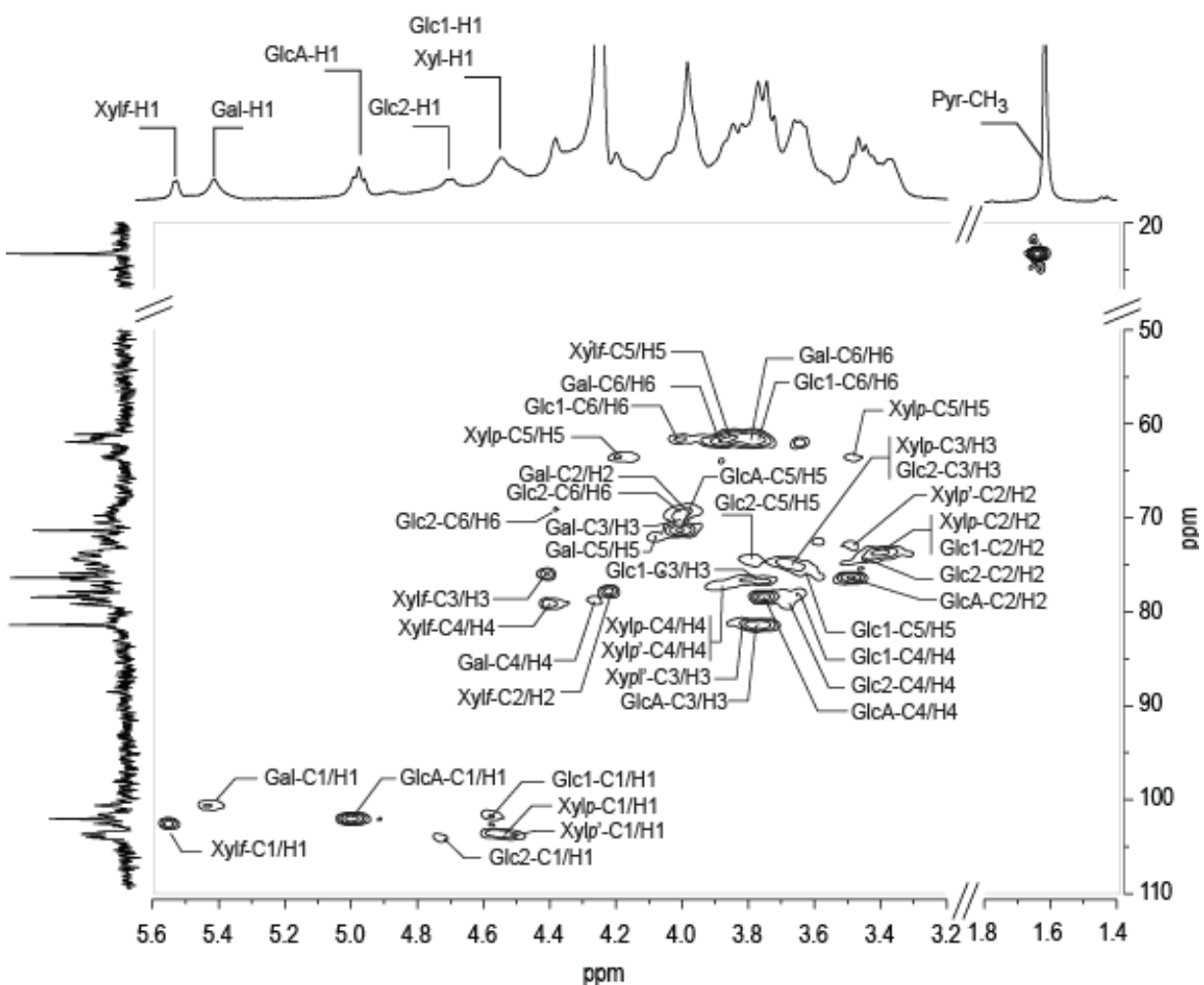


Figure 1. Heteronuclear $^1\text{H}/^{13}\text{C}$ chemical shift correlation (HSQC) spectrum of the polysaccharide secreted by *Nostoc commune*. The spectrum was recorded at 353 K

197 Two glucose residues could be distinguished in the spectra (Glc1-C1/H1: 100.90/4.57 ppm and Glc2-
 198 C1/H1: 103.33/4.71 ppm), one galactose residue (Gal-C1/H1: 99.91/5.42 ppm) and three xylose residues
 199 were observed: two in pyrano configuration (Xyl_p-C1/H1: 102.97/4.49 ppm and Xyl'_p-C1/H1:
 200 102.75/4.54 ppm) and one in furano configuration (Xylf-C1/H1: 101.86/5.54 ppm). The glucuronic acid
 201 residue (GlcA-C1/H1: 101.34/4.99 ppm) was systematically decorated by a pyruvate group which could
 202 be removed by a mild acid treatment conducting to the shift of the anomeric proton from 4.99 to 4.57 ppm
 203 (Figure S4). Investigations of the linkages using heteronuclear ¹H/¹³C chemical shift correlation (HMBC)
 204 revealed strong similarities with the previous work of Helm and co-workers (2000) on the polysaccharides
 205 of *N. commune* DRH-1. The repeating unit seemed very similar except that the glucuronic residue was
 206 decorated by a pyruvate group instead of lactate (named nosturonic residue) and that the ribofuranoside
 207 residue was replaced by a xylofuranoside residue.

208 **Table 2:** ¹H and ¹³C NMR chemical shifts recorded on the secreted polysaccharide of *Nostoc commune*.

Sugar Residue		1	2	3	4	5	6 (6a,6b) Pyr (CH ₃ , C, CO)	
Polymer								
Glc-1 →4)-β-D-Glcp-(1→+	¹ H	4.57	3.39	3.78	3.65	3.58	3.98, 3.78	
	¹³ C	100.90	72.97	76.00	76.87	74.91	60.74	
Xyl_p →4)-β-D-Xylp-(1→	¹ H	4.49	3.40	3.65	3.86	4.17, 3.47		
	¹³ C	102.97	73.00	73.74	76.01	63.00		
Glc-2 →4,6)-β-D-Glcp-(1→	¹ H	4.71	3.43	3.66	3.68	3.78	4.34, 4.00	
	¹³ C	103.33	73.56	74.07	78.07	73.56	69.09	
Pyr-GlcA β-D-(2,3Pyr)-Glc _p A (1→	¹ H	4.99	3.48	3.76	3.74	4.00	no	1.64
	¹³ C	101.34	75.61	80.70	77.95	70.64	174.90	22.59, 109.03, 175.65
Gal →4)-α-D-Galp-(1→	¹ H	5.42	4.00	4.01	4.27	4.06	3.87, 3.77	
	¹³ C	99.91	69.02	69.79	78.07	71.30	61.22	
Xylp' →3,4)-β-D-Xylp-(1→	¹ H	4.54	3.48	3.80	3.88	4.17, 3.47		
	¹³ C	102.75	71.77	79.87	76.01	62.70		
Xylf α-D-Xylf-(1→	¹ H	5.54	4.21	4.40	4.39	3.82		
	¹³ C	101.86	77.13	75.22	78.46	60.40		
Depyruvated Polymer								
dPyr β-D-GlcpA-(1→	¹ H	4.57	3.42	3.58	3.58	3.77	no	
	¹³ C	102.63	72.83	75.45	71.71	76.18	175.75	

209

210 **3.3 Analyses of the enzymatic degradation products of the *N. commune* polysaccharide**

211 To deepen the structural characterization of the polysaccharide, we took advantage of our recent
 212 discovery of an endo-acting enzyme able to cleave the polysaccharides in oligosaccharides (Helbert et al.,
 213 2019). This new enzyme which is the first member of the glycoside hydrolase family GH160 to cleave the

214 linkage between the galactose (sub-site -1) and a glucose (sub-site +1) residues giving oligosaccharide
 215 series with galactose at the reducing end and glucose at the non-reducing end.

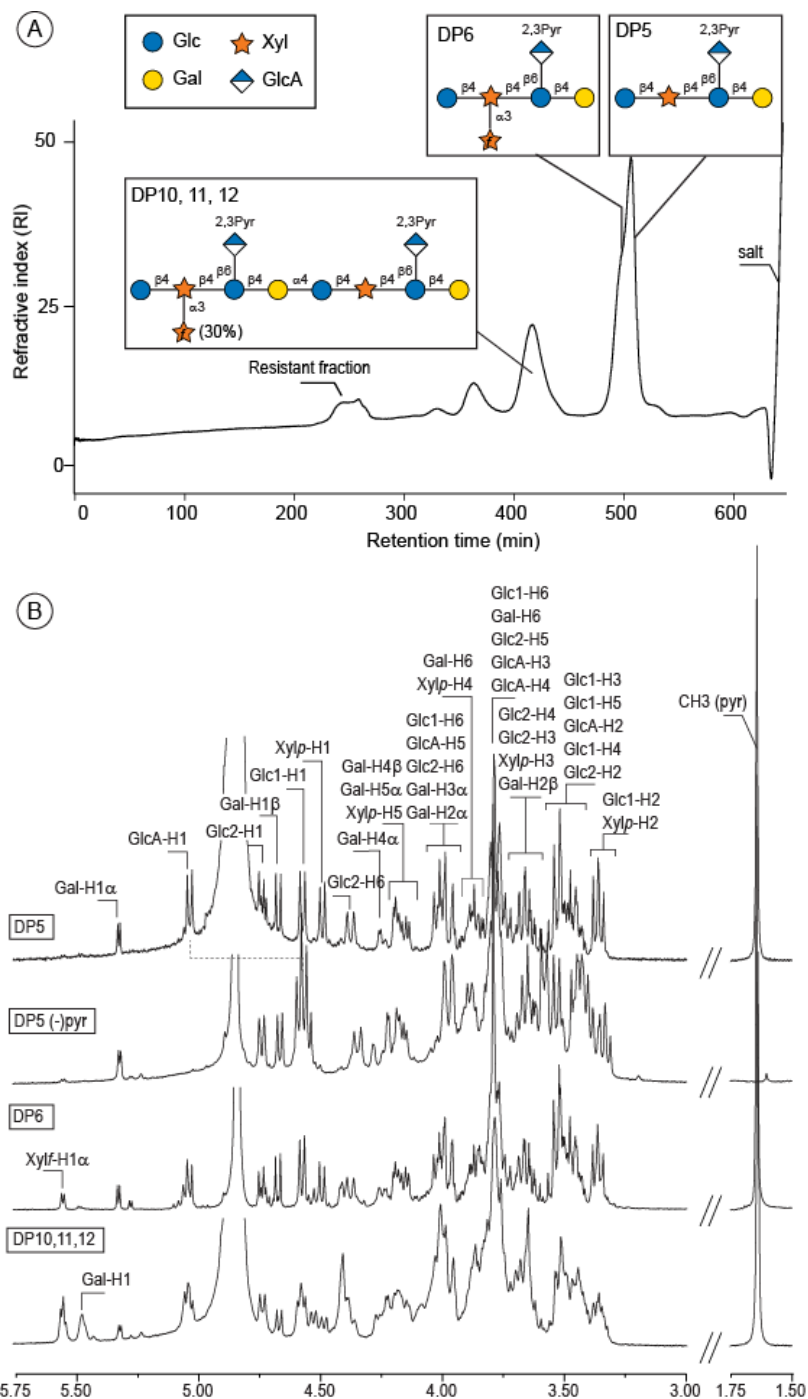


Figure 2. Purification and characterization of the enzymatic degradation products of *N. commune* polysaccharide. **A)** Size exclusion chromatography of the degradation products. Inset: the structure of the oligosaccharides determined by NMR analyses. **B)** ¹H NMR spectra of the oligosaccharides isolated after purification by chromatography. The spectra were recorded at 293 K

217 **Table 3:** ^1H and ^{13}C NMR chemical shifts of the oligosaccharides obtained after enzymatic degradation of
 218 the *N. commune* polysaccharide. Only the chemical shifts of the DP6 and longer oligosaccharides which
 219 were different from those of DP5 and depyruvated DP5 were reported in the table. ^a: Glc2→Gal α /
 220 Glc2→Gal β values.

Sugar Residue		1	2	3	4	5	6 (6a,6b)	pyr (CH ₃ , C, CO)
DP5								
Glc-1 β -D-Glcp-(1→	^1H	4.57	3.36	3.53	3.47	3.52	3.98, 3.78	
	^{13}C	101.07	72.79	75.41	69.58	75.90	60.70	
Xylp →4)- β -D-Xylp-(1→	^1H	4.49	3.36	3.65	3.88	4.16, 3.49		
	^{13}C	103.06	73.08	73.64	76.20	62.88		
Glc-2 →4,6)- β -D-Glcp-(1→	^1H	4.74/4.73 ^a	3.44	3.66	3.72	3.73	4.37, 4.01	
	^{13}C	103.35/103.49 ^a	73.45	74.06	77.99	73.48	68.07	
Pyr-GlcA β -D-(2,3Pyr)-GlcA-(1→	^1H	5.03	3.52	3.76	3.76	4.01	no	1.64
	^{13}C	101.29	75.59	80.72	78.47	70.57	175.33	22.56, 108.94, 175.99
Galα →4)- α -D-Galp	^1H	5.32	3.95	4.01	4.26	4.19	3.87, 3.77	
	^{13}C	92.27	68.69	69.58	78.47	69.96	61.15	
Galβ →4)- β -D-Galp	^1H	4.67	3.62	3.82	4.19	3.79	3.87, 3.77	
	^{13}C	96.38	72.07	73.16	77.25	74.47	61.29	
Depyruvated DP5								
Glc-1 β -D-Glcp-(1→	^1H	4.59	3.34	3.54	3.47	3.52	3.98, 3.78	
	^{13}C	101.09	72.78	75.48	69.55	75.93	60.67	
Xylp →4)- β -D-Xylp-(1→	^1H	4.55	3.39	3.65	3.90	4.17, 3.44		
	^{13}C	102.91	73.01	73.74	76.40	62.87		
Glc-2 →4,6)- β -D-Glcp-(1→	^1H	4.75	3.44	3.66	3.77	3.73	4.35, 3.98	
	^{13}C	103.50/103.59 ^a	73.47	74.06	77.86	73.47	67.95	
GlcA β -D-GlcA-(1→	^1H	4.57	3.42	3.56	3.52	3.77	no	
	^{13}C	102.63	72.83	75.48	71.82	76.33	175.81	
Galα →4)- α -D-Galp	^1H	5.33	3.94	4.01	4.29	4.19	3.88, 3.78	
	^{13}C	92.28	68.70	69.55	78.44	69.84	60.92	
Galβ →4)- β -D-Galp	^1H	4.67	3.62	3.82	4.23	3.79	3.88, 3.78	
	^{13}C	96.39	72.10	73.17	77.36	74.31	61.10	
DP6								
Xylp →3,4)- β -D-Xylp-(1→	^1H	4.54	3.44	3.82	3.88	4.16, 3.49		
	^{13}C	103.02	72.26	79.87	76.20	62.68		
Xylf α -D-Xylf-(1→	^1H	5.55	4.23	4.40	4.40	3.82		
	^{13}C	101.88	76.87	74.90	78.47	60.54		
DP10, 11, 12								
Glc-1 →4)- β -D-Glcp-(1→	^1H	4.59	3.39	3.81	3.70	3.64	3.98, 3.78	
	^{13}C	100.88	72.80	76.00	76.87	74.91	60.68	
Gal →4)- α -D-Galp-(1→	^1H	5.47	4.00	4.01	4.22	4.11	3.87, 3.77	
	^{13}C	99.80	68.88	69.72	78.07	71.16	61.04	

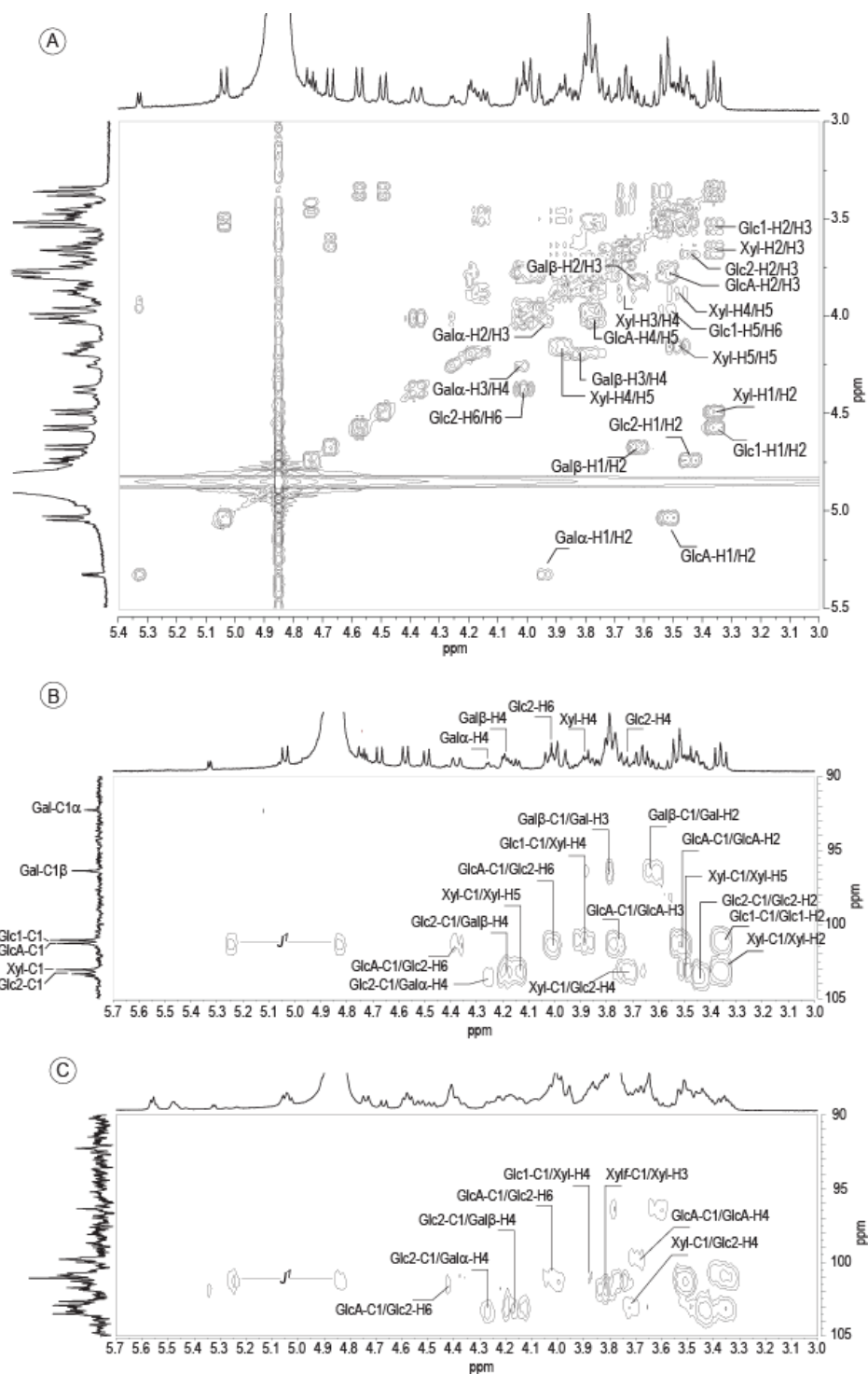


Figure 3. Analyses of the oligosaccharides by NMR. A) COSY spectrum of DP5. B) Detail of the HMBC spectrum of DP5. C) Detail of the HMBC spectrum of DP10, 11, 12. The spectra were recorded at 293 K.

223 Enzymatic degradation of the polysaccharide was monitored by size exclusion chromatography
224 and oligosaccharides obtained after complete digestion of the macromolecule were collected (Figure 2A).
225 Composition of the oligosaccharide fractions was determined (Figure S5) and their structure characterized
226 by NMR (Figure 2B). The peak eluting at 507 min contained two oligosaccharides which could be
227 identified by collecting different fractions of this peak. Based on composition analyses, ^{13}C and ^1H NMR
228 spectra, the smallest oligosaccharide was found to have a degree of polymerization of DP=5 (elution time
229 510 min) with galactose at the reducing end and glucose at the non-reducing end as expected.

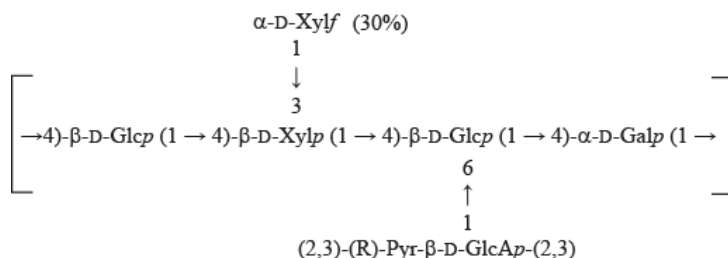
230 After mild acid hydrolysis, allowing the removal of the pyruvate group, the chemical shift of the
231 glucuronic acid residue was shifted from C1/H1: 101.29/5.03 ppm to C1/H1; 102.63/4.57 ppm (Figure 2B,
232 spectra DP5(-)Pyr). Analysis of the correlation system in the COSY (Figure 3A) allowed attributing the
233 chemical shifts to five residues: the galactose located at the reducing end (Gal α -C1/H1: 92.27/5.32 ppm
234 and Gal β -C1/H1: 96.38/4.67 ppm), the glucose residue at the non-reducing end (Glc1nr-C1/H1:
235 101.07/4.57 ppm), the glucuronic acid residue decorated by a pyruvate (GlcA-C1/H1: 101.29/5.03 ppm), a
236 second glucose residue (Glc2-C1/H1 \rightarrow Gal α : 103.35/4.74 ppm and Glc2-C1/H1 \rightarrow Gal β : 103.49/4.73 ppm)
237 and a xylopyranoside residue (Xyl-C1/H1: 103.06/4.49ppm) (Table 3). The linkage between these
238 residues were determined straightforwardly using heteronuclear $^1\text{H}/^{13}\text{C}$ chemical shift correlation (HMBC,
239 Figure 3B). Altogether the structure of the oligosaccharide deduced from these analyses was a
240 pentasaccharide made of the tetrasaccharide: β -D-Glc (1 \rightarrow 4)- β -D-Xyl (1 \rightarrow 4)- β -D-Glc (1 \rightarrow 4)- α / β -D-
241 Gal, branched with a 2,3pyruvated β -D-GlcA linked at the position 6 of the internal glucose residue.

242 We noticed that the two glucose residues observed in the main chain presented very different
243 chemical shifts. The residue Glc1nr-C1/H1: 101.07/4.57 ppm showed a lower C1/H1 chemical shifts than
244 for the residue Glc2 (Glc2-C1/H1 \rightarrow Gal α : 103.65/4.74 ppm and Glc2-C1/H1 \rightarrow Gal β : 103.49/4.73 ppm).
245 The resolution of the NMR spectra recorded on the oligosaccharides and, more especially, the
246 carbon/proton coupling observed in heteronuclear single quantum coherence (HSQC) were obvious. The
247 lower Glc1-C1/H1 chemical shifts is explained by its bounding to a xylose residue as previously observed
248 by Helm and co-workers (2000). Calculating of the chemical shifts using the online CASPER program
249 (<http://www.casper.organ.su.se/casper/>; Lundborg and Widmalm, 2011) gave Glc1-C1/H1= 101.94/4.59
250 ppm and Glc2-C1/H1 = 104.48/4.65 ppm which values were close to those recorded.

251 The asymmetric center of the pyruvate group was determined by NOESY (Nuclear Overhauser
252 Effect Spectroscopy) according to the same strategy reported by Severn & Richards (1992). The NOESY
253 spectrum revealed a long distance correlation between the protons of the methyl group of pyruvate (Pyr-
254 CH₃= 1.64 ppm) with proton H2 of the glucuronic residues (GlcA-H2=3.52 ppm). No correlation was
255 observed with proton H3 demonstrating the pyruvate group adopted the R configuration.

256 Composition analyses of the fractions containing higher size oligosaccharides were enriched in
 257 xylose residues (Figure S5) suggesting the branching of xylose residues on the main chain as observed for
 258 the undigested polysaccharide. The fraction collected at 495 min, contained an oligosaccharide which
 259 NMR spectra and chemical shifts recorded in ^1H and ^{13}C were almost identical to that of the previous DP5
 260 suggesting a very similar structure (Figure 2B, DP6). However, additional signals observed in NMR
 261 spectra (Xylf-C1 = 101.88 ppm, Xylf-H1 = 5.55 ppm) were attributed to an additional residue suggesting
 262 the occurrence of an oligosaccharide of DP=6. Comparison and of the DP5 and DP6 spectra, as well as the
 263 proton correlation system evidenced by the well-resolved COSY spectra and the $^1\text{H}/^{13}\text{C}$ chemical shift
 264 correlation highlighted by the HMBC spectra allowed determining straightforwardly the chemical shifts
 265 and the coupling constants. The data recorded were in agreement with an α -xylofuranoside residue and
 266 similar to interpretations reported in previous works (Angyal, 1979; Kocharova et al., 1994). Other
 267 furanoside residues not detected in composition analyses could also be excluded after NMR
 268 investigations. Indeed, the expected C4/H4 = 84.7 ppm/4.11 ppm of α -ribofuranose (Helm et al., 2000),
 269 the H4 = 3.68 ppm of α -mannofuranose (Angyal, 1979) or C4/H4 = 82 ppm / 3.71 ppm of α -
 270 galactofuranoside (Katzenellenbogen et al. 2012) were too different to the recorded C4/H4 = 78.47
 271 ppm/4.40 ppm attributed to the α -xylofuranoside. Signals of the heteronuclear $^1\text{H}/^{13}\text{C}$ chemical shift
 272 correlation (HMBC) were too weak to determine unambiguously the linkage of the residue.

273 The peak eluting at 415 min was a mixture of several oligosaccharides which were not isolated
 274 and which sizes were estimated to DP10, 11 or 12, corresponding to different combinations of the DP5
 275 and DP6. Based on the signal attribution of the DP5 and DP6, most of the ^1H and ^{13}C NMR signals could
 276 be ascribed (Table 3). More interestingly, the linkage between the Glc and Gal residues cleaved by the
 277 enzymes was determined using heteronuclear $^1\text{H}/^{13}\text{C}$ chemical shift correlation (HMBC, Figure 3C). The
 278 correlation between the Gal-C1 and Glc-H4 observed in the spectrum, demonstrated unambiguously the
 279 α -D-Gal (1 \rightarrow 4)-D-Glc linkage. The linkage of the xylofuranoside residue was also well identified in the
 280 HMBC spectra and revealed the residue was bound to the xylopyranoside residue of the main chain by
 281 (1,3) linkage. The repeating unit of the polysaccharide deduced from the detailed analyses of the
 282 oligosaccharide and the the unmodified polymer was:



283

284 3.4 Dispersion and diversity of *Nostoc commune* polysaccharides

285 After an exhaustive survey of the literature, the structures of the polysaccharides secreted by *Nostoc*
286 strain were presented in Figure 4. The polysaccharide secreted by the *Nostoc* strain isolated at Saint
287 Martin d'Uriage (France, Europe) showed very similar structural characteristic with those secreted by
288 several Asian strains (Figure 4). The polysaccharide of *Nostoc commune* strains isolated in Mongolia,
289 China or Japan shared a tetra-saccharide repeating unit: $[\rightarrow 4) \beta\text{-D-Glc (1} \rightarrow 4)\text{-}\beta\text{-D-Xyl (1} \rightarrow 4)\text{-}\beta\text{-D-Glc$
290 $(1 \rightarrow 4)\text{-}\alpha\text{-D-Gal (1} \rightarrow]$, systematically branched at position 6 of a glucose residue by a β -linked
291 glucuronic acid residue. No decoration of the glucuronic acid residue was reported for the polysaccharide
292 secreted by the Chinese strain (Brüll et al., 2000). A lactate group was observed in the case of the
293 polysaccharide of the Mongolian origin (Helm et al., 2000) instead of the pyruvate group found in our
294 studied polysaccharide. Interestingly, both lactate and pyruvate were identified in the polysaccharides of a
295 strain isolated in Japan (Kajiyama et al., 2001) suggesting that derivatization of the glucuronic acid
296 residue by lactate or pyruvate can co-occur in *Nostoc commune*. The occurrence of only one specific
297 group - pyruvate or lactate - may reflect some adaptation to the environment or are an independent
298 evolution of the strains which could have abandoned one derivatization pathway. Also it can't be excluded
299 that specific modification of the polysaccharides may be caused by biotic interactions such as, for
300 example, co-existing bacterial strains.

301 Another difference observed is the replacement of the ribose residue found in the Mongolian
302 strain polysaccharide by a xylose residue in our study, adopting in both cases the furano configuration. In
303 a biosynthesis point of view, the replacement a D-ribose by L-xylose its epimer at position 3 residue is not
304 biochemically improbable and could proceed from an evolution of the biosynthetic pathway. Composition
305 analyses of polysaccharides secreted by various strains of *N. commune* show that in addition to ribose and
306 xylose, other residues such as arabinose, fucose or rhamnose could occur along the polysaccharide chain
307 (Huang, Liu, Paulsen & Klaveness 1998; De Philippis, Ena, Paperi, Sili & Vincenzini, 2000). Altogether,
308 its seems that the polysaccharides secreted by *Nostoc commune* strains isolated in various place in the
309 world have conserved structural characteristic which suggested a selection pressure on the biosynthetic
310 pathway leading to the preservation of mandatory biological and physico-chemical properties. The
311 capacity of the gelatinous substance embedding of the cyanobacterial cells composed of the
312 polysaccharide, associated to another component such as proteins, is probably its capacity to swell/de-
313 swell as a function of the amount of water in a reversible way. Implication of the polysaccharide in the
314 defence mechanisms as a mechanical or biological barrier to protect akinete resting cells probably played
315 also a role in maintaining the structural properties of the polysaccharide.

316 *N. commune* strain investigated in this study and the strains isolated in the desert of Mongolia (*N.*
317 *commune* DRH-1) were identified using genetic tools. Few other structural data on the polysaccharides

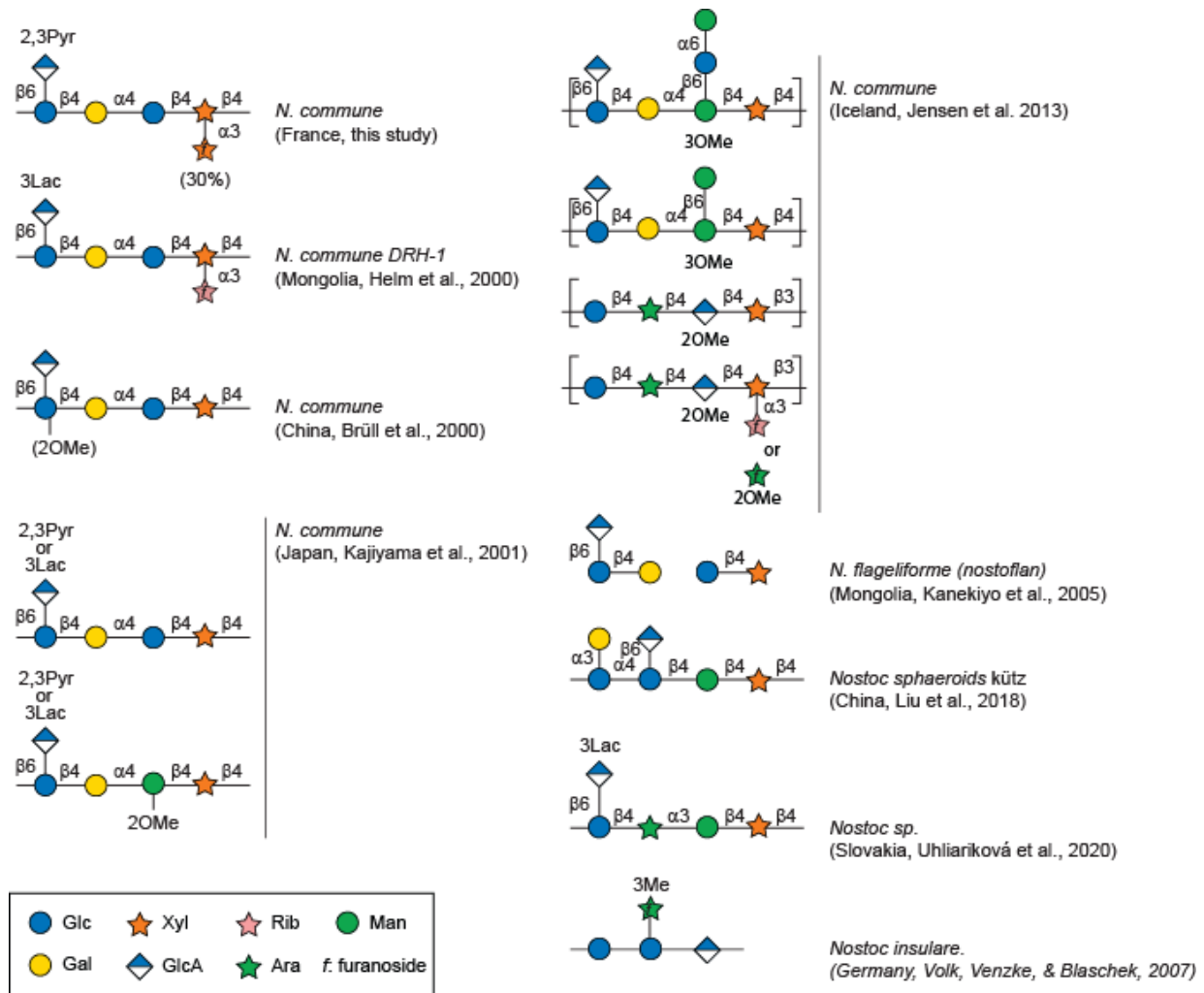


Figure 4. Comparison of the structures of the polysaccharides secreted by *Nostoc* sp. strains isolated in various places in the world.

318 secreted by other species of *Nostoc* were also reported. However, genetic data were lacking and
 319 identification of the species was based on morphology considerations. In this context, comparison of the
 320 polysaccharide structures in regard to the evolution of *Nostoc* species is therefore difficult. However, the
 321 oligosaccharides obtained after acid hydrolysis of the polysaccharide of a strain identified as *Nostoc*
 322 *flagelliforme* (Mongolia, Kanekiyo et al., 2005) suggested also a conserved carbohydrate backbone with
 323 the same pentasaccharide sequence. *Nostoc commune* can form filament depending on growth condition
 324 and it is then difficult to discriminate some *Nostoc commune* var *flagelliforme* with true *Nostoc*
 325 *flagelliforme*. Structure of reported *Nostoc* sp. polysaccharides presented in Figure 4 suggested that the
 326 repeating unit made of a penta-saccharide seemed common probably reflecting a common biosynthesis
 327 template which evolved in the *Nostoc* lineages.

328

329 **4. Conclusion**

330 The structure of the polysaccharide secreted by the strain of *N. commune* isolated in Saint Martin
331 d'Uriage (France) was resolved. Comparison of our results with previously characterized structure of
332 polysaccharide of *N. commune* strains found in Europe and Asia suggested the conservation of a
333 pentasaccharide template reflecting a conservation of the biosynthesis pathway. The building block made
334 of the tetrasaccharide [\rightarrow 4)- β -D-Glc (\rightarrow 4)- β -D-Xyl (\rightarrow 4)- β -D-Glc (\rightarrow 4)- α -D-Gal (\rightarrow),
335 systematically branched by a β -linked glucuronic acid, presented lactate and pyruvate derivatives
336 according to the origin of the *Nostoc commune* strain. Also, we noticed that a mannose or methylated
337 mannose glucose residue was found in place of a glucose residue of the main chain. The structure of *N.*
338 *flageliforme* polysaccharides showed was similar to *N. commune* suggesting a common biosynthesis
339 pathway ancestor. The important role of the polysaccharide in the growth and survival of the
340 cyanobacterial cells supposes that strong alteration of modification of the structure and, therefore, the
341 associated physico-chemical properties will be detrimental to the cells. The conservation of the
342 polysaccharide structure of the world-wide spread *N. commune* strains probably reflects a strong selection
343 pressure on the structure of the polysaccharides.

344

345 **Acknowledgments**

346 This work was supported by the French National Research Agency (Grant ANR-14-CE06-0017). W.H.,
347 E.M and A.A have received support from the Glyco@Alps Cross-Disciplinary Program (Grant ANR-15-
348 IDEX-02), Labex ARCANE, and Grenoble Graduate School in Chemistry, Biology, and Health (Grant
349 ANR-17-EURE-0003). E.M. and A.A. are supported by Grant Alpalga ANR-20-CE02-0020.

350

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450

451 Supporting information

452 **Supplementary Figures**

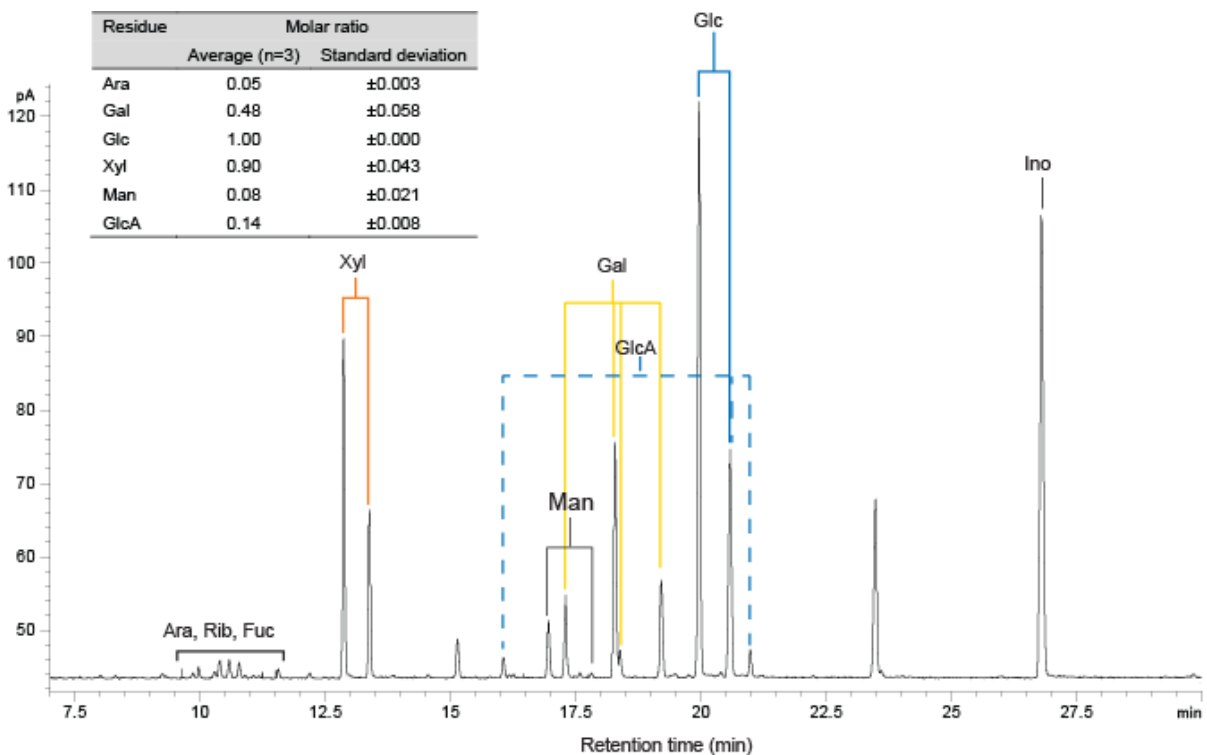
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456 **Figure S1:** Gas chromatogram recorded on the hydrolysis products of the *Nostoc commune*
457 polysaccharide. Inset: Molar ratio of the various detected residues.

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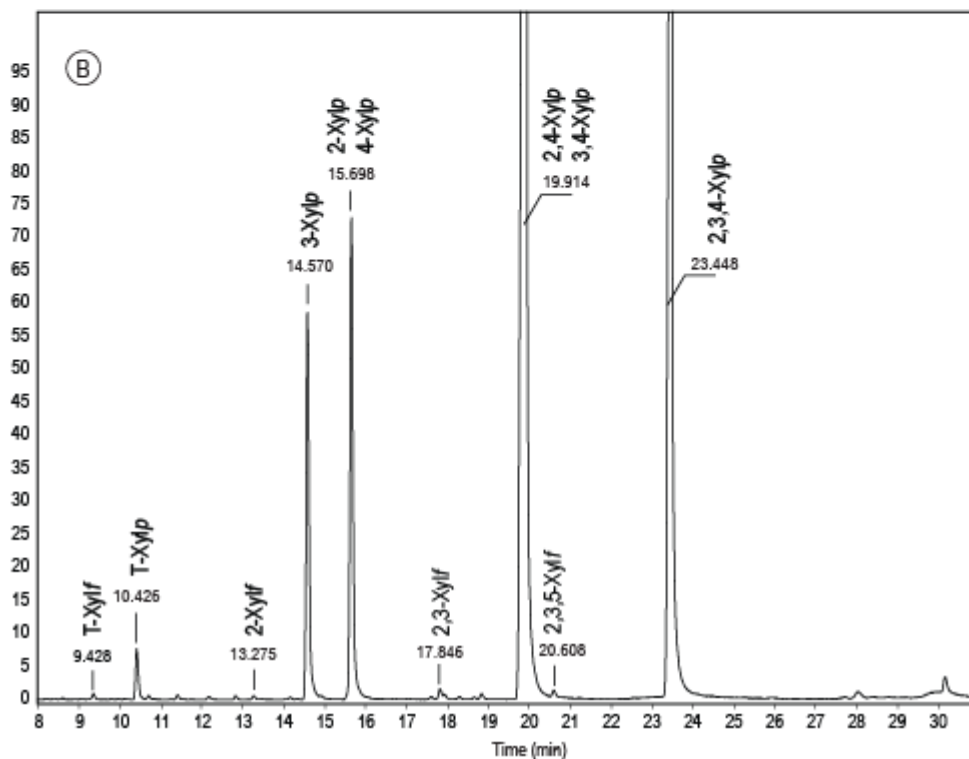
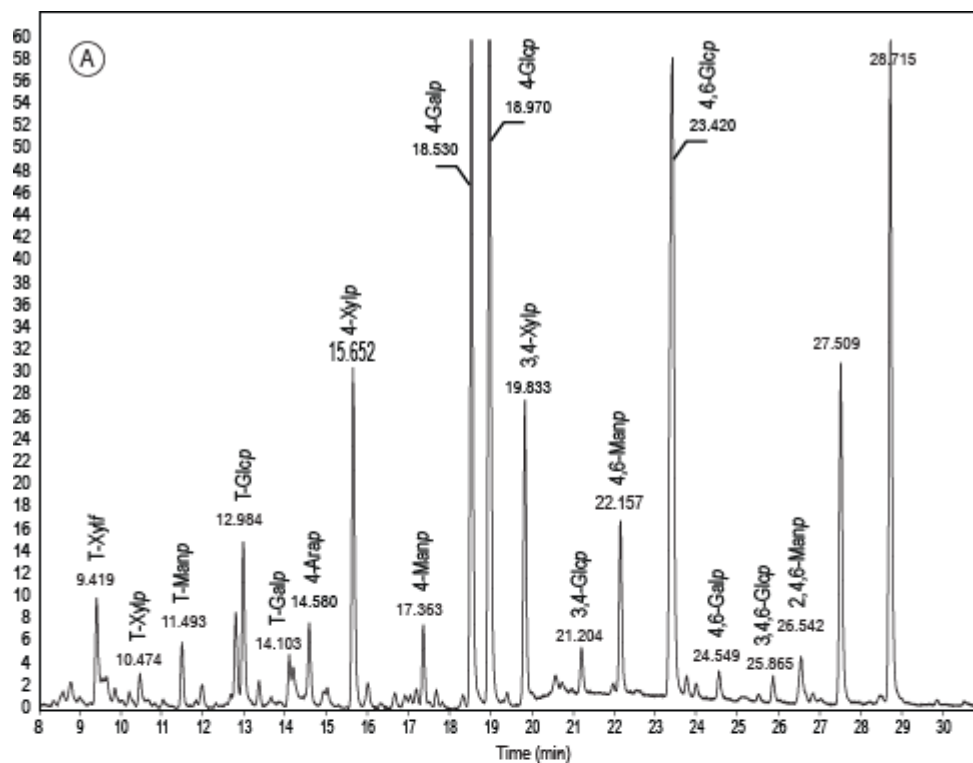
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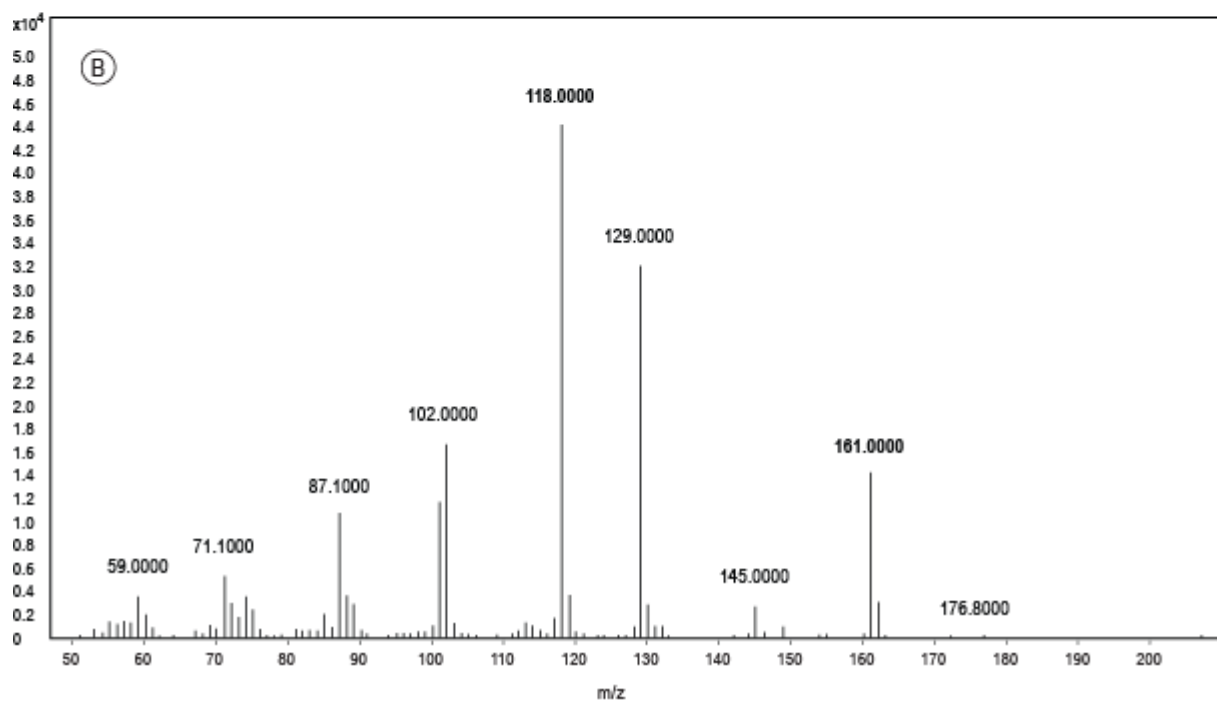
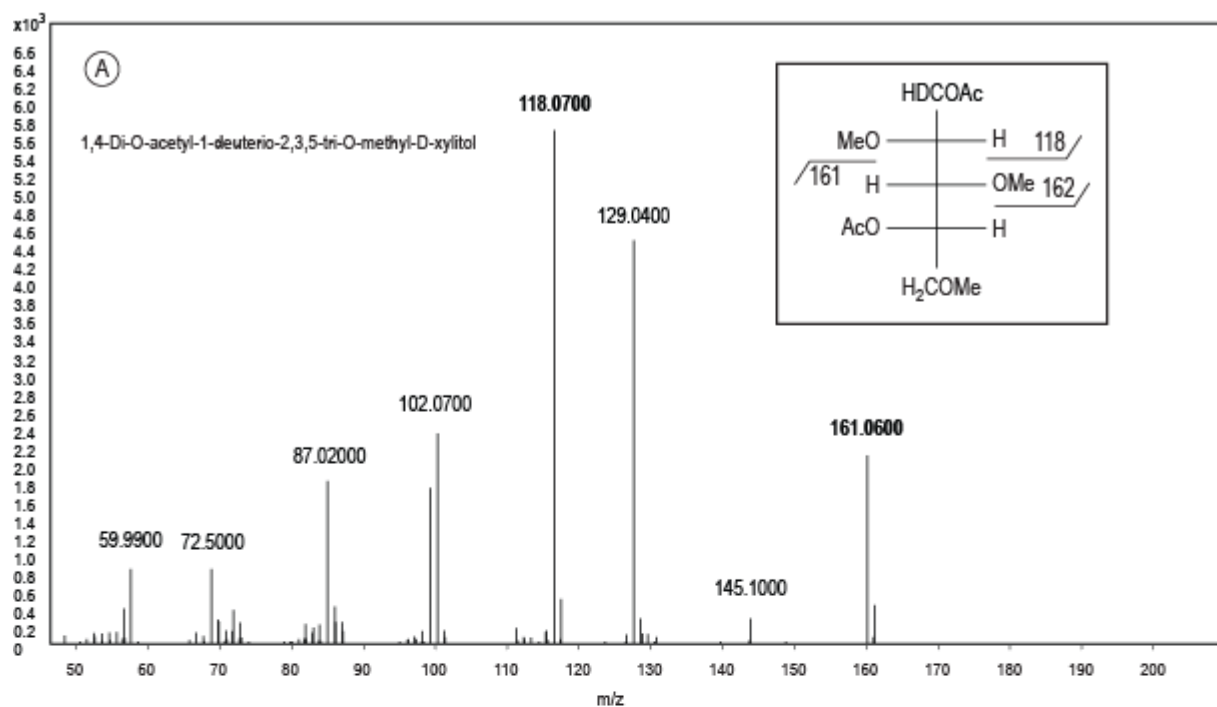
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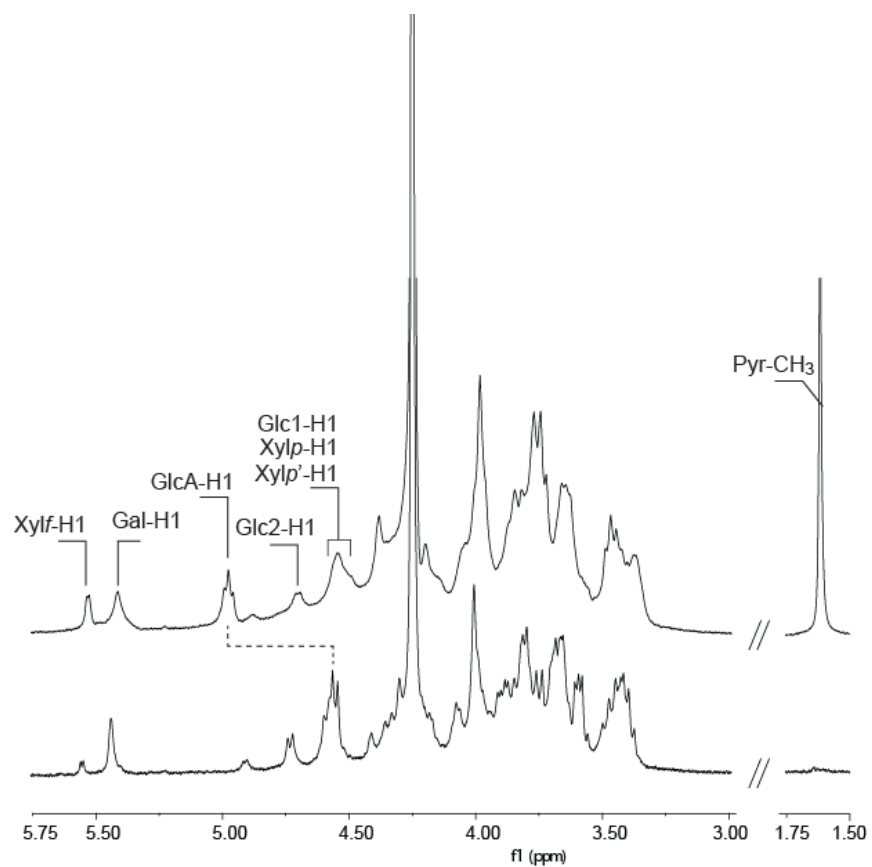
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467 **Figure S2:** Gas chromatogram recorded on the permethylated residues of the *Nostoc commune*
 468 polysaccharide (A) and the xylose standard residues (B).

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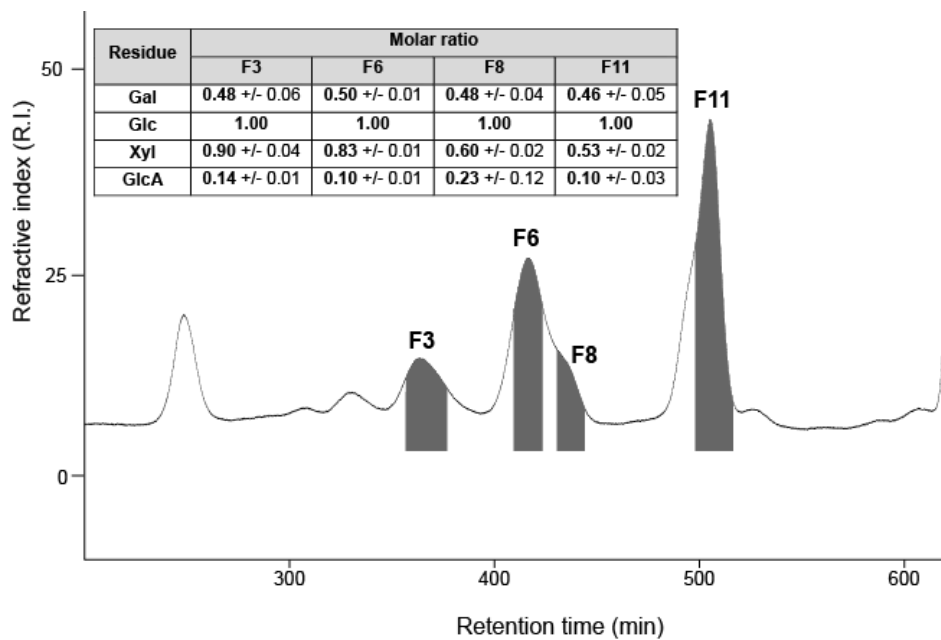


470
 471 **Figure S3:** (A) Fragmentation product of the 1,4-di-O-acetyl-1-deuterio-2,3,5-tri-O-methyl-D-xylitol
 472 eluting at 9.468 min in the gas chromatogram of the xylose derivatives standard (Figure S3A). (B)
 473 Fragmentation products obtained on the derivatives eluting at 9.419 in the gas chromatogram recorded on
 474 the *N. commune* polysaccharide (Figure S3B)
 475



476
 477 **Figure S4.** ^1H NMR spectra of the *Nostoc commune* polysaccharides. Top: The purified polysaccharide.
 478 Bottom: The polysaccharide was subjected to mild acid treatment. The spectra were recorded at 353 K

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 485 **Figure S5:** Size exclusion chromatogram recorded on the enzymatic degradation products of *N. commune*
 486 polysaccharide. The composition of the oligosaccharides collected were determined and reported in the
 487 inserted table.
 488