Biocatalytic quantification of α -glucan in particulate marine organic matter

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Abstract

Marine algae drive the marine carbon cycle, converting carbon dioxide into organic material. A major component of this produced biomass is a variety of glycans; and yet their chemical composition and individual involvement in production, sedimentation and bacterial uptake remain largely unknown due to a lack of analytical tools for glycan-specific quantification.

Marine α -glucans include a range of storage glycans from red and green algae, bacteria, fungi and animals. Although these compounds are likely to account for a high amount of the carbon stored in the oceans they have not been quantified in marine samples so far.

Here we present a method to extract and quantify α -glucans in particulate organic matter from algal cultures and environmental samples using a sequential physicochemical extraction and enzymes as α -glucan-specific probes. This enzy-

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15 matic assay is more specific and less susceptible to side reactions than chemical
16 hydrolysis. Using HPAEC-PAD to detect the hydrolysis products allows for a
17 glycan quantification in particulate marine samples even at low concentration of
18 $\approx 2\text{-}7\text{ }\mu\text{g/L}$ α -glucans.

19 We measured α -glucans (and compared their concentration with the β -glucan
20 laminarin) in three microalgae laboratory cultures as well as in marine particulate
21 organic matter from the North Sea and western North Atlantic Ocean. While
22 laminarin from diatoms and brown algae is an essential component of marine
23 carbon turnover, our results further indicate the significant contribution of starch-
24 like α -glucans to marine particulate organic matter.

25 Henceforth, the combination of glycan-linkage-specific enzymes and chro-
26 matographic hydrolysis product detection can provide a powerful tool in the
27 exploration of marine glycans and their role in the global carbon cycle.

28 **1. Introduction**

29 Autotrophic organisms in the oceans are estimated to contribute about 46 %
30 of the global primary production, converting carbon dioxide to organic compounds
31 (Field et al., 1998). Major components of this biomass are glycans (Myklestad,
32 1974). While most of the marine glycans are consumed and eventually recon-
33 verted into carbon dioxide, marine glycans can also be a carbon sink and become
34 part of the ocean floor sediment. This carbon storage capacity makes marine
35 glycans an important subject for environmental - and particularly climate-related
36 - research (Engel et al., 2004; Hedges et al., 2001).

37 Glycans are complex and often non-linear polymers built from numerous pos-

sible monosaccharides (sometimes with chemical modifications) and linkage possibilities (Laine, 1994). This complexity makes the structural analysis of glycans challenging and the analysis of glycans in marine dissolved or particulate organic matter even more difficult as the glycans are present in complex mixtures and at often low individual concentrations. The lack of structure-specific analytical tools for marine glycan samples leads to these glycans being commonly identified and quantified by their monosaccharide content after chemical hydrolysis (Engel and Händel, 2011; Panagiotopoulos and Sempéré, 2005). This non-selective acid hydrolysis destroys information about glycosidic linkages and some chemical modifications. It is therefore not suitable to unequivocally identify different types of glycans. This results in a fundamental lack of knowledge about the structures and functions of marine polysaccharides in the marine carbon cycle (Hedges et al., 2001).

Glucans are glycans derived from D-glucose residues. Some glucans like starch, glycogen and laminarin are energy storage glycans while other glucans like cellulose are structural polymers (Suzuki and Suzuki, 2013; Painter, 1983). Acid hydrolysis makes these glycans of different biological origins indistinguishable and obscures insight into their roles in the marine carbon cycle.

α -Glucans include a range of different types of polysaccharides, most of them containing α -1,4 and α -1,6 glycosidic linkages. Starch is a mixture of the linear α -1,4-linked amylose and the branched amylopectin composed of α -1,4- and α -1,6-glycosidic bonds (Imberty et al., 1991). Starch is commonly known as a storage glycan for terrestrial plants but it is also found in green algae. Floridean starch is a storage α -glucan in red algae similar to amylopectin but with a higher degree of α -1,6-branching. Glycogen is another highly α -1,6-branched storage

63 α -glucan found in animals, fungi and bacteria (Ball and Morell, 2003). Although
64 the amount of α -glucans in the ocean is unknown, analysis of marine bacterial
65 polysaccharide degradation pathways (Fang et al., 2019; Kappelmann et al.,
66 2019) indicate that α -glucans are an important carbohydrate source for marine
67 bacteria.

68 Glycan specific glycoside hydrolases (GHs) can be applied to identify and
69 quantify α -glucans in the ocean. There are several well-characterized starch
70 specific GHs (α -amylases and amyloglucosidases) that have been used to quantify
71 starch in food (McCleary et al., 1994, 2002), yet a more sensitive method for
72 starch quantification in marine environmental samples is missing.

73 In this study, a set of commercial enzymes was adapted into an assay for quan-
74 tification of α -glucans in different types of microalgae and unprocessed marine
75 environmental samples. This assay was tested in parallel with a previously devel-
76 oped laminarin assay (Becker et al., 2020) on different types of α -glucans and
77 other polysaccharides to explore possible side reactions and compare the enzy-
78 matic hydrolysis (EH) with the commonly used acid hydrolysis (AH). Additionally,
79 the detection range of the assay was tested using two types of glucose detection,
80 the spectrophotometric PAHBAH assay and the chromatographic HPAEC-PAD.
81 Furthermore, the extraction of glucans from different types of microalgae was
82 optimized. The α -glucan and laminarin content of these microalgae was tested
83 at different days of algal growth and compared to the total particulate organic
84 carbon (POC) in these samples. Finally, the assay was used on two sets of par-
85 ticulate organic matter (POM) samples from the North Sea (spring 2020) and
86 the western North Atlantic Ocean (spring 2019) to demonstrate that this method
87 allows for a quantification of low concentrations of α -glucans alongside laminarin

88 in crude marine samples.

89 2. Methods

90 2.1. Algal cultures

91 Three species of microalgae were used as examples for marine organic material
92 to test different extraction protocols and enzymatic hydrolysis of algal glucans:
93 The green microalga *Ostreococcus tauri* (Ral et al., 2004) was grown in L1
94 medium (Guillard, 1983; Guillard and Hargraves, 1993), and the red microalgae
95 *Porphyridium purpureum* and the diatom *Thalassiosira weissflogii* were grown in
96 ESAW medium (Harrison et al., 1980). Algae cultures were kept at a constant
97 temperature of 15°C, with a 12-h/12-h light/dark cycle, without stirring and
98 irradiated with $\approx 140 \mu\text{mol photons m}^{-2}\text{s}^{-1}$.

99 For glucan extraction tests, duplicate T75 flasks with 400 mL growth medium
100 were inoculated with 5 mL of 7-days old algal cultures. After 20 days the material
101 was filtered at 200 mbar on a combusted (450 °C, 4 h) 25 mm GF/F glass
102 microfiber filter (using 20 mL of algal culture per filter). The filters were stored
103 at -20°C until extraction.

104 For the α - and β -glucan quantifications algae were cultivated in triplicate
105 batch cultures. A total volume of 250 mL in T75 suspension cell culture flasks
106 was inoculated with 5 mL of a 25-mL culture that had been grown for 7 days.
107 10-25 mL of each culture was taken over 20 days and filtered as described above.

108 2.2. Environmental sample collection

109 Sampling in the North Atlantic Ocean (40°53.7'N, 60°11.9'W) was carried
110 out in May 2019 and in the North Sea (54°11.3'N, 7°54.0'E) in April 2020. In

both locations, surface seawater was collected and directly filtered through pre-combusted (400 °C, 4 h) GF/F glass filters with 142 mm diameter (Whatman glass microfiber filters, WHA1825142, Sigma-Aldrich). For the Atlantic Ocean samples, 50 L seawater were filtered through each GF/F filter with a peristaltic pump (Watson Marlow 630 S) at 40 rpm. Filters were stored at -80 °C until further analysis. For the North Sea samples, 15 L seawater were filtered through each GF/F filter with an air pressure pump (Flojet G57, ITT Industries) at $\approx$ 0.2-0.5 bar. Filters were stored at -30 °C until further analysis.

2.3. Extraction of glucans from particulate algae material

The filters were cut into equally sized pieces and subjected to extraction tests. One filter piece was kept as a non-extracted reference. Each extraction was tested using filter-part triplicates of three different filters. Afterwards the extracted and non-extracted filter-parts were put under AH conditions and the glucose content of the acid extract tested using HPAEC-PAD. The extraction conditions tested include water or 1M NaOH extractions at 99°C or in a sonication bath (SONOREX SUPER RK 510, Bandelin), different extraction times and sequential extractions.

2.4. Acid hydrolysis

Glycan samples in solution or algal samples on glass fiber filters were incubated in 1 M HCl for 24 h at 100°C in sealed glass ampoules to chemically hydrolyze glycans. Afterwards, 100 μ L of each acid solution was evaporated using a speed-vac (RVC 2-18 CDplus HCl resistant, Christ) and resuspended in 100 μ L buffer or Milli-Q water.

134 **2.5. Enzymatic hydrolysis**

135 α -Glucans were hydrolyzed using α -amylase (*Aspergillus oryzae*) obtained
136 from Megazyme (Product code: E-ANAAM) and amyloglucosidase (*Aspergillus*
137 *niger*) from Merck (Product code: 10115). The stock concentration was 1 U/ μ L
138 for both enzymes. Samples containing polysaccharide standards or extracted
139 glucans were split into six subsamples: three for enzyme hydrolysis and three for
140 non-hydrolyzed controls.

141 Triplicate subsamples of 90 μ L aqueous glycan extract were mixed with 10
142 μ L sodium acetate buffer (1 M, pH 4.5), 0.4 μ L α -amylase stock (in 3.2 M
143 ammonium sulphate), 0.9 μ L amyloglucosidase stock (in 0.1 M sodium acetate)
144 and 1 μ L BSA solution (100 mg/mL). Triplicate non-hydrolyzed subsamples were
145 prepared in the same way, but contained 0.4 μ L 3.2 M ammonium sulphate and
146 0.9 μ L 0.1 M sodium acetate instead of enzymes. Reaction contents were mixed
147 and incubated for 35 min at 50 °C (and 400 rpm shaking) in a heat block. Then,
148 the enzyme reactions were inactivated 5 min at 99 °C, centrifuged (10.000 rpm
149 for 30 s) and cooled on ice.

150 Subsamples used for laminarin quantification (90 μ L) were hydrolyzed using
151 90 μ L aqueous glycan solution, 10 μ L 500 mM MOPS buffer (pH 7.0) and
152 laminarinases as previously described (Becker et al., 2017).

153 **2.6. PAHBAH reducing sugar assay**

154 Photometric quantification of polysaccharide hydrolysis products was per-
155 formed using the PAHBAH reducing sugar assay (Lever, 1972). One milliliter of a
156 freshly prepared 9:1 (v/v) mixture of PAHBAH reagent A (0.3 M 4-hydroxybenz-
157 hydrazide, 0.6 M HCl) and PAHBAH reagent B (48 mM trisodium citrate, 10 mM
158 CaCl₂, 0.5 M NaOH) was added to 0.1 mL of sample. After incubation for 5

159 min at 99 °C, the samples were cooled on ice and the absorbance at 410 nm
160 was measured using 10 mm pathlength semimicro cuvetts and a BioSpectrom-
161 eter (Eppendorf). Glucose standards were prepared and measured in the same
162 manner.

163 **2.7. HPAEC–PAD**

164 All samples for HPAEC–PAD (High-performance Anion Exchange Chromatog-
165 raphy with pulsed amperometric detection) were filtered using a 0.2 µm Spin-X
166 filter and transferred into an HPLC-vial with a micro-insert. The monosaccha-
167 ride (and specifically glucose) content was determined using HPAEC-PAD with a
168 Dionex CarboPac PA10 column (Thermo Scientific) and monosaccharide mixes
169 as standards for calibration (Engel and Händel, 2011). The oligosaccharide con-
170 tent was determined using Dionex CarboPac PA200 and PA100 columns (Thermo
171 Scientific) and appropriate malto- and laminarin oligosaccharides (Megazyme).

172 **2.8. Glucan quantification**

173 Glucans in extracted samples were quantified as glucose equivalents based on
174 a calibration curve generated with glucose standards.

175 The glucose signals were inferred from PAHBAH absorbance readings or inte-
176 grated peak areas from HPAEC-PAD chromatograms. Each hydrolyzed sample or
177 non-hydrolyzed control was measured in triplicates. To correct for background
178 signals in the enzymatically digested samples, the glucose values detected for
179 non-hydrolyzed samples were subtracted. No correction was applied for total
180 glucan quantification after acid hydrolysis.

181 The glucan concentrations in filter extracts were normalized by extraction
182 volume, filter size and filtration volume.

183 **2.9. Particulate organic carbon quantification**

184 Particulate organic matter from microalgae cultures or environmental sam-
 185 ples were both filtered on glass fiber filters. Defined pieces of these filters were
 186 punched out in triplicates and subjected to an acidic atmosphere with concen-
 187 trated HCl for 24 h in a desiccator to remove inorganic carbon. Afterwards, the
 188 filters were dried for 24 h at 60 °C and wrapped in combusted tin foil. The car-
 189 bon quantification was performed by an elemental analyzer (vario MICRO cube;
 190 Elementar Analysensysteme) using sulfanilamide as a calibration standard.

191 **3. Assessment**

192 **3.1. Optimal conditions for enzymatic α -glucan hydrolysis**

193 Based on an assay for amylose and amylopectin quantification in cereal starches
 194 and flours (Megazyme, 2018), a similar assay for total α -glucan quantification
 195 (without amylose/amylopectin separation) was developed and optimized for a
 196 sample volume of 100 μ L, a starch concentration of 100 μ g/mL (from corn,
 197 Sigma-Aldrich) and hydrolysis product detection using the PAHBAH reducing
 198 sugar assay (Lever, 1972). Optimal EH was achieved by incubating a 100 μ L
 199 starch sample in 100 mM sodium acetate buffer (pH 4.5) for 35 min at 50 °C
 200 in the presence of 0.4 U of the *endo*-acting α -amylase (from *Aspergillus oryzae*,
 201 Megazyme) and 0.9 U of the *exo*-acting amyloglucosidase (from *Aspergillus niger*,
 202 Merck). Under these conditions, PAHBAH reducing sugar signals were maximal
 203 (Figures S1, S2). Subsequently, the complete hydrolysis of different α -glucan
 204 polysaccharides, namely starch, glycogen, amylose or amylopectin down to glu-
 205 cose monosaccharides was confirmed by HPAEC-PAD (Figure S3).

208

209 **Figure 1: Enzymatic hydrolysis is more effective than acid hydrolysis for**
 210 **starch:** Enzymatic hydrolysis (EH) products of starch, acid hydrolysis (AH)
 211 products of starch, non-digested starch, and glucose as calibration standard were
 212 measured in α -glucan assay buffer (100 mM sodium acetate buffer, pH 4.5) using
 213 the PAHBAH reducing sugar assay. $A_{410\text{nm}}$ signals above 1.9 were measured in
 214 diluted samples. Error bars denote standard deviation ($n=3$, technical replicates).
 215 Solid lines represent regression lines.

206 3.2. Enzymatic hydrolysis of α -glucans is more effective than acid hy- 207 drolysis

217 The enzymatic α -glucan hydrolysis was tested on different starch concentra-
 218 tions and compared to AH using the PAHBAH reducing sugar assay as a glucose
 219 detection method (Figure 1). The limit of quantification was found to be ≈ 11
 220 $\mu\text{g/mL}$ starch, both for acid hydrolysis, and for enzymatic hydrolysis in combi-
 221 nation with the PAHBAH assay. However, it was found that the EH of starch (1
 222 - 1000 $\mu\text{g/mL}$) generated a higher photometric signal than AH suggesting that
 223 a more complete polysaccharide hydrolysis was achieved or that EH is less prone

224 to generate side products (Cai et al., 2014). Especially at higher starch concen-
225 trations above 250 $\mu\text{g}/\text{mL}$ the AH glucose signal visibly flattened, resulting in a
226 nonlinear regression line. In contrast, EH of starch followed a linear regression
227 model for all measured starch concentrations up to 1 mg/mL .

228 A complete hydrolysis of 1 g of a glucan into glucose would theoretically
229 yield approximately 1.1 g of glucose by the addition of 1 water molecule per
230 hydrolyzed glucose molecule. In comparison, Figure 1 shows that even EH is not
231 complete, as 1 mg/mL starch yield 0.6 mg/mL of glucose equivalents. This result
232 is probably due to the water insolubility of some starch particles, and because
233 the amount of enzymes added was optimized for 100 $\mu\text{g}/\text{mL}$ starch samples.

234 It should be noted that for these measurements, the glucose calibration curve
235 was prepared in water. Under AH conditions the glucose signals in the calibration
236 curve were ($\approx -20\%$) lower (Figure S4). These data indicate that lower glucose
237 signals in acid hydrolyzed starch samples could partially be due to acid catalyzed
238 conversion of glucose. But as this loss does not account for the total difference
239 in signal of acid and enzymatic starch hydrolysis, our results also indicate that
240 AH of starch is less complete than EH.

241 Overall, these results show that AH - the traditional quantification method
242 - may underestimate the true glycan content in environmental samples due to
243 incomplete glycan hydrolysis and possible side reactions reducing the detectable
244 monosaccharide content.

245 **3.3. Enzymatic hydrolysis of α -glucans is specific**

255 To explore the specificity of the enzymatic α -glucan assay compared with AH,
256 14 commercially available polysaccharides were tested. The enzymatic laminarin
257 assay was included as a control. Figure 2 shows the reducing sugar assay results

246

247 **Figure 2: Enzymatic hydrolysis of α-glucans is specific and more effec-**
 248 **tive than acid hydrolysis:** Hydrolysis efficiency of different polysaccharides
 249 (100 μg/mL) using acid hydrolysis (AH) compared to enzymatic hydrolysis (EH)
 250 using the α-glucan or laminarin assay. All tested polysaccharides are listed in
 251 Table S1. Only the results of polysaccharides that could be hydrolyzed by either
 252 of the enzymatic assays are depicted. Data points represent individual samples,
 253 and error bars denote standard deviation (n=3, technical replicates).

258 for the 8 polysaccharides that could be hydrolyzed by either of the enzymatic
259 assays compared to AH. Additional tested polysaccharides that could only be
260 hydrolyzed by acid are listed in Table S1.

261 To compare the measured glucans, glucose was used as a quantification stan-
262 dard. The glucose standards used for quantification were treated the same way
263 as the respective samples. This corrects for absorbance changes in the assay
264 caused by addition of specific buffers and enzymes, or by AH (Figure S4).

265 The α -glucan assay is able to hydrolyze all polysaccharides containing α -
266 glucan 1-4- and 1-6-linkages, namely amylopectin, amylose, glycogen and pul-
267 lulan. The reducing sugar signal of enzymatically hydrolyzed amylopectin and
268 glycogen equals approximately 100 $\mu\text{g}/\text{mL}$ glucose indicating a complete hydroly-
269 sis to glucose. Amylose is poorly soluble in water and therefore only 60 $\mu\text{g}/\text{mL}$
270 glucose equivalents, presumably produced by shorter-chain amylose, could be
271 measured using EH.

272 Similar to the results in Figure 1 the concentration of glucose hydrolysis prod-
273 ucts is often higher for the α -glucan enzymatic assay than for the AH with the
274 notable exception of pullulan, which is less effectively hydrolyzed by the enzy-
275 matic assay than by AH. Pullulan is comprised of α -1-4-maltotriose units linked
276 by α -1-6-linkages and should - in theory - be completely hydrolyzed by the ap-
277 plied *endo*-1-4- α -amylase and *exo*-1,4/1,6- α -amylglucosidase. However, as the
278 observed reducing sugar signal is only slightly increased by the application of
279 these enzymes, it can be assumed that this α -amylase needs a longer uninter-
280 rupted α -1-4-linked glucose chain to be more active. Thus, the α -glucan assay
281 can mainly be used to identity and quantify starch-like α -glucans with 1-4 chains
282 and optional 1-6 branches.

283 While the laminarin assay is effective on both laminarins tested and the
284 amount of released glucose is similar and slightly higher than than for AH, the
285 only other polysaccharide being partially hydrolyzed by this assay is lichenan. As
286 lichenan is comprised of β -1-3 and β -1-4 linked glucose units and some of the
287 applied laminarinases have a β -1-3 activity (Becker et al., 2017), this small side
288 reaction is to be expected. However, the results show that the laminarin assay
289 cannot detect β -glucan from barley and any of the tested α -glucans.

290 The relatively high reducing sugar signal of non-hydrolyzed laminarin from
291 *Laminaria digitata* indicates the presence of shorter polysaccharide chains. In
292 contrast, non-hydrolyzed polysaccharide from other batches of the same laminarin
293 had no measureable reducing sugar signal (Becker et al., 2020). This further
294 underlines the advantage in measuring glycans in glucose equivalents over using
295 commercial polysaccharides as standards.

296 Overall, these results demonstrate that a combination of the enzymatic as-
297 says can distinguish the important storage glucans; starch-like α -1,4/1,6-glucans
298 and laminarin - something that cannot be achieved using traditional chemical
299 hydrolysis.

300 **3.4. Microalgal glucans can be extracted using hot water and sonication**

315 The use of enzymatic assays to quantify glucans in marine POM requires that
316 the glucans can be extracted from environmental samples. Particulate microalgal
317 material was used to test different extraction protocols. The extraction efficiency
318 was determined by measuring the ratio of glucose detected by HPAEC-PAD on
319 extracted and non-extracted filter pieces after AH conditions, assuming that these
320 conditions are sufficient for a complete glucan extraction and hydrolysis. For the
321 extraction of glucans from filters, we tested different combinations of water and

301

302 **Figure 3: α -Glucans can be extracted from microalgae:** Residual glucose
303 content of algal POM on glass fiber filters after extractions (see below) and
304 subsequent AH was determined using HPAEC-PAD. (A) Hot water extraction (1
305 h, 99 °C) of *T. weissflogii* (*T. w.*) and *O. tauri* (*O. t.*) POM. (B) *O. tauri*
306 extractions with a longer incubation time (water, 2 h, 99 °C), sequential double
307 extraction (water, 2 x 1 h, 99 °C), alkaline extraction (1 M sodium hydroxide,
308 1 h, 99 °C) or sequential water (1 h, 99 °C) and alkaline (1 M sodium hydroxide,
309 1 h, 99 °C) extraction. (C) Hot water extraction of microalgae (*T. weissflogii*,
310 *O. tauri*, *P. purpureum* = *P. p.*) with prior sonication treatment (1 h sonication
311 bath, 1 h, 99 °C), (D) additional subsequent alkaline extraction (in 1 M sodium
312 hydroxide), 1 h sonication bath, 1 h, 99 °C. Individual data points are shown in
313 darker colors, error bars denote standard deviation (n=3, technical replicates).

322 NaOH incubations with, and without, sonication.

323 Figure 3 shows the results of these extraction condition tests as the proportion
324 of (total) glucose that remains in the filters after extraction. Water extraction
325 for 1 h at 99 °C is able to extract more than 90 % of *T. weissflogii* particulate
326 glucans. For *O. tauri*, only up to 50 % of glucans are extracted under the same
327 conditions (Figure 3 A). A longer incubation time, sequential water extractions or
328 a hot alkaline extraction using 1 M sodium hydroxide (1 h, 99 °C) do not increase
329 the extraction efficiency for *O. tauri*. However, a subsequent additional extraction
330 using 1 M sodium hydroxide (1 h, 99 °C) decreases the amount of residual glucose
331 to approximately 30 % (Figure 3 B). Sonication of water extracted samples before
332 incubation at 99 °C gives similar results; approximately 25 % residual glucose for
333 *O. tauri* and 30 % residual glucose for *P. purpureum* (Figure 3 C).

334 The extraction was slightly improved when using 1 h sonication in 1 M NaOH
335 followed by 1 h at 99 °C. This treatment resulted in residual glucose values of 3 %,
336 20 % and 25 % in *T. weissflogii*, *O. tauri* and *P. purpureum*, respectively (Figure
337 3 D). However, this additional alkaline extraction did not substantially increase
338 the efficiency of the extraction protocol. Therefore, POM samples were extracted
339 using hot water (1 h, sonication; 1 h, 99 °C) in the following sections. The in-
340 tegrity of laminarin and starch was also tested and confirmed by EH of starch and
341 laminarin standards that had been subjected to extraction conditions compared
342 to non-subjected samples.

343 These results demonstrate that glucans in *O. tauri* and *P. purpureum* are
344 more resistant to aqueous extraction methods than glucans in *T. weissflogii*.
345 This result is probably due to different glucans present in these species. While
346 material of the diatom *T. weissflogii* should contain laminarin (Becker et al.,

2017), the green microalgae *Ostreococcus tauri* and the red microalgae *Porphyridium purpureum* should contain starch and flouridean starch respectively (Sorokina et al., 2011; Sheath et al., 1979). Laminarin is highly soluble in water, whereas α -glucans vary in their water solubilities; branched α -glucans like amylopectin and glycogen have a high solubility and linear amylose has a low solubility. The differences in extraction efficiency should be taken into account with regard to glucan quantifications as the true content might be higher.

3.5. α -Glucans and laminarin can be quantified in parallel in particulate matter extracts from cultivated microalgae

Glucan quantification in POM from *T. weissflogii*, *O. tauri* and *P. purpureum* laboratory cultures expressed as glucose equivalents. Laminarin and α -glucans were quantified in microalgal POM extracts using enzymatic hydrolysis and subsequent PAHBAH reducing sugar assay (EH, green and blue symbols). Total glucan was quantified by acid hydrolysis and subsequent PAHBAH reducing sugar assay (AH, magenta symbols). The amount of glucose equivalents in each sample was calculated using glucose calibrations curves.

To investigate the proportion of α - and β -glucans in the aforementioned three microalgae, enzymatic α -glucan and laminarin hydrolysis assays have to be used. Three non-axenic cultures of each of the microalgae *O. tauri*, *P. purpureum* and *T. weissflogii* were grown in the laboratory and samples from each culture were taken over 20 days and filtered onto glass fiber filters.

Glycans were extracted from these filters using sonication and hot water (as described above) and hydrolyzed by AH or EH. Figure 4 shows the glucose equivalents as determined by the reducing sugar assay over the course of 20 days of algal growth.

Figure 4: α -Glucans can be quantified alongside laminarin in microalgal extracts using glucan specific enzymatic hydrolysis: Glucan quantification in POM from *T. weissflogii*, *O. tauri* and *P. purpureum* laboratory cultures expressed as glucose equivalents. Laminarin and α -glucans were quantified in microalgal POM extracts using enzymatic hydrolysis and subsequent PAHBAH reducing sugar assay (EH, green and blue symbols). Total glucan was quantified by acid hydrolysis and subsequent PAHBAH reducing sugar assay (AH, magenta symbols). The amount of glucose equivalents in each sample was calculated using glucose calibrations curves. Error bars denote standard deviation (n=3, biological replicates).

Laminarin was detectable in *T. weissflogii* diatom cultures after 6 days, but could not be detected in cultures of green microalgae *O. tauri* and red microalgae *P. purpureum* over the 20 days incubation period. Conversely, α -glucans were detected in *O. tauri* and *P. purpureum* from day 4 or 2, respectively. Overall, these findings match the polysaccharide profiles of these three microalgae (Becker et al., 2017; Sorokina et al., 2011; Sheath et al., 1979). However, a small amount of α -glucans could also be measured in two of the three *T. weissflogii* cultures on day 10 and 20. As the algal cultures were non-axenic, it is possible that these α -glucans originate from bacteria.

Overall, AH produces a similar total glucose signal as both the enzymatic assays combined. Since the PAHBAH assay would be reactive to all reducing sugars released by AH this indicates that α -glucans and laminarin (a β -glucan) are the predominant glycans in these algal species and under the culture conditions and duration used here.

Figure 5 shows the glucan quantification on day 20 in relation to the total POC. It should be noted that POC was quantified directly on glass fiber filters without prior extraction, whereas for glucan quantification the filters were extracted by sonication and hot water. As this extraction might not be complete, the true glucan (especially α -glucan-) content might be higher than depicted.

For *T. weissflogii* about 40 % of the total POC can be assigned to laminarin. Furthermore, approximately 10 % of POC are detectable in two of three cultures as reducing sugars released by AH. These 10 % are hydrolyzed by enzymatic α -glucan hydrolysis and probably originate from bacterial glycogen. About 40 % of the POC of *O. tauri* corresponds to α -glucans. In this case, the reducing sugars hydrolyzed by acid equal the enzymatically hydrolyzed α -glucans. The POC of

398

399 **Figure 5: α -Glucans account for a substantial amount of the total POC**
 400 **in red and green algae:** α -Glucan and laminarin quantification of microalgal
 401 POM from *T. weissflogii*, *O. tauri* and *P. purpureum* laboratory cultures after
 402 20 days of growth. Glucan quantities are shown as glucose equivalent carbon
 403 relative to the total POC. Error bars denote standard deviation (n=3, biological
 404 replicates).

417 *P. purpureum* after 20 days of algal growth can largely be contributed to glycans
418 as approximately 80 % of POC are AH products. However, only about 60 % of
419 *P. purpureum* POC is hydrolyzed by the α -glucan assay. This discrepancy might
420 be due to a higher content of monomeric glucose or other glucose containing
421 oligo- and polysaccharides.

422 In summary, around 40-60 % of POC in the tested microalgal cultures can
423 be ascribed to glucose coming from glucans. The glucans in *T. weissflogii* are
424 mostly laminarin (β -glucans), the glucans in *O. tauri* and *P. purpureum* are
425 mostly starch-like α -glucans.

435 **3.6. α -Glucans and laminarin can be quantified in parallel in marine** 436 **environmental particulate organic matter**

437 Marine environmental POM samples were taken from the western North At-
438 lantic Ocean (40°53.7'N, 60°11.9'W) during May 2019 and in the North Sea
439 (54°11.3'N, 7°54.0'E) near Helgoland (Germany) during April 2020. In both
440 cases, POM in surface water was filtered onto 0.7 μ m glass fiber filters. The POC
441 content was quantified directly from non-extracted filter-circle-cutouts, while the
442 glucan content was determined using AH or EH of hot water extracts of quarter
443 filters. The glucose content in the samples was found to be too low for quan-
444 tification by the photometric reducing sugar assay. Therefore, HPAEC-PAD was
445 used to determine the glucose content in hydrolyzed and non-hydrolyzed extract
446 samples. With HPAEC-PAD, as little as 0.3 μ g/mL and 0.2 μ g/mL glucose
447 could be detected following AH or EH, respectively, of a starch standard sample.
448 In comparison, the quantification limit for the reducing sugar PAHBAH assay
449 was 11 μ g/mL.

450 HPAEC-PAD analysis of enzymatically hydrolyzed α -glucans and laminarin

426

427 **Figure 6: α -Glucans can be quantified alongside laminarin in marine**
 428 **POM samples using glucan specific enzymatic hydrolysis:** POM samples
 429 on 0.7 μm glass fiber filters were extracted and hydrolyzed using acid hydrolysis
 430 (AH) (magenta symbols) or enzymatic hydrolysis (EH) (blue and green symbols).
 431 Square symbols represent samples from the western North Atlantic Ocean (May
 432 2019). Triangle symbols represent samples from the North Sea (April 2020).
 433 Error bars denote standard deviation (n=3, technical replicates).

451 shows that the only product is glucose (Figure S3). Therefore glucose can be
452 used as a standard to quantify and compare α -glucans and laminarin. Similar
453 chromatograms of hydrolyzed and non-hydrolyzed extracts from algal cultures
454 and environmental samples are depicted in Figure S5.

455 Figure 6 shows the results of glucan quantification of environmental POM
456 samples (taken at different times over 2-3 days) in relation to the total POC.
457 While laminarin content in POM samples from the western North Atlantic Ocean
458 varies between 2-16 $\mu\text{g/L}$ carbon, it is higher in most of the North Sea samples
459 (4-36 $\mu\text{g/L}$ carbon from laminarin). This is in agreement with the abundance of
460 microalgae at the time of sampling: Samples from the Atlantic Ocean were har-
461 vested in the morning (10:30) and evening (22:30) in oligotrophic waters, while
462 North Sea samples were taken in the morning (7:30) and evening (21:30) in May
463 2020 during the phytoplankton spring bloom. For these North Sea samples a
464 difference between higher α -glucan and laminarin levels in the evening ($\text{POC} >$
465 $300 \mu\text{g/L}$) and lower levels in the morning ($\text{POC} < 300 \mu\text{g/L}$) is observed. This
466 could indicate a diel cycling of α -glucans similar to laminarin during phytoplank-
467 ton blooms (Becker et al., 2020). This difference in glucan levels depending on
468 time could not be observed in samples from the western North Atlantic Ocean.

469 Overall, less α -glucan than laminarin was detected in the environmental sam-
470 ples (2-7 $\mu\text{g/L}$, West Atlantic and 1-4 $\mu\text{g/L}$, North Sea). The combined glucose
471 value for laminarin and α -glucan, determined with EH, is similar to the total
472 glucose value determined with AH. This observation is consistent with the re-
473 sults shown above (Figures 4 and 5). This result further indicates that most
474 glucose containing polysaccharides are detected by one of the two enzymatic as-
475 says. Laminarin content in POM appears to correlate with increasing total POC

476 ($R^2 = 77.5 \%$) - in accordance with previous studies (Becker et al., 2020) -,
 477 whereas α -glucan content is significantly lower and apparently non-correlating
 478 with total POC ($R^2 = 9.4 \%$). It should be noted that these α -glucans may
 479 originate not only from starch-producing red and green algae, but also from
 480 glycogen-producing bacteria.

481 **4. Discussion**

482 A method for the detection and quantification of starch-like α -glucans in
 483 the ocean has been developed and applied to microalgal cultures and marine
 484 environment samples. Combined with enzymatic laminarin hydrolysis most of
 485 the glucans in marine POM can be quantified.

486 This method has been shown to be highly specific and robust. However,
 487 the assay cannot distinguish between the different types of α -1,4/1,6-glucans
 488 and is therefore not suitable to determine the origin of the detected α -glucans.
 489 To investigate this question further the degree of α -1,6-branching could also be
 490 determined by expanding the assay to include an *endo*-acting α -1,6-isoamylase
 491 (Figure S1). Knowing the average degree of α -1,6-branching in POM glucans
 492 does not unequivocally identify the type of detected α -glucans, but it might
 493 allow for an estimate if the main α -glucan is the highly branched glycogen. An-
 494 other approach to identify α -glucan types might be the application of separation
 495 techniques based on different solubilities.

496 A related issue requiring further consideration is the hot water (combined with
 497 ultrasonication) extraction applied in this study. Varying water solubilities of dif-
 498 ferent α -glucans and the presence of starch granules in algae cells impede a com-
 499 plete glucan extraction from microalgal POM. Previous studies where starch was

500 extracted from algae material also employed ultrasonication (Kobayashi et al.,
501 1974) or other cell disrupting techniques like hot alkaline extraction, bead-beating
502 (Wong et al., 2019) and grinding (Yu et al., 2002). However, for these studies
503 large amounts (kg range) of algae material were used and their aim was rather
504 a high purity of starch extracts and not a complete extraction. Our method of
505 hot water extraction combined with ultrasonication from POM is probably not
506 complete for all types of α -glucans, but it can be employed for small quantities
507 of algae material and it allows parallel detection of other polysaccharides by en-
508 zymatic assays. Nevertheless, it is possible that the true α -glucan content of the
509 environmental POM samples is higher than detected.

510 Furthermore, this work has shed light on problems of glucan quantification
511 after chemical lysis, as this method consistently produced less glucose products
512 from polysaccharide standards than enzymatic hydrolysis. Despite this problem,
513 acid glycan hydrolysis allows for a complete and non-specific hydrolysis and can
514 provide valuable information on monosaccharide composition of environmental
515 samples.

516 α -Glucan concentrations in marine surface water POM have - in our examples
517 - been shown to be lower than laminarin but at a constant level, independent
518 of increased POC concentrations during the seasonal algal bloom in the North
519 Sea 2020. Higher α -glucan concentrations are to be expected near coastal and
520 estuary areas, due to input of terrestrial plants and sites with distinct green
521 and red algal growth. However, it is likely that for marine glycan samples α -
522 glucan concentrations will usually be lower than laminarin concentrations. This
523 low concentration of specific types of glycans in the marine carbon pool is a
524 major obstacle for marine glycan analysis and requires the application of adequate

525 detection methods with low detection limits like HPAEC-PAD or MS.

526 In conclusion our study has shown that enzymes can be used to identify and
527 quantify glycans in parallel in marine particulate organic matter. The combination
528 of laminarin and α -glucan assays allows for a complete glucan detection in these
529 samples.

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541 **6. Author contributions**

542 N.S. designed and conducted experiments. S.V.-M. and M.S.-J. took envi-
543 ronmental samples. J.-H.H. supervised the project. All authors edited the paper.

References

- Ball, S.G., Morell, M.K., 2003. From bacterial glycogen to starch: understanding the biogenesis of the plant starch granule. *Annual review of plant biology* 54, 207–233.
- Becker, S., Scheffel, A., Polz, M.F., Hehemann, J.H., 2017. Accurate quantification of laminarin in marine organic matter with enzymes from marine microbes. *Appl. Environ. Microbiol.* 83, e03389–16.
- Becker, S., Tebben, J., Coffinet, S., Wiltshire, K., Iversen, M.H., Harder, T., Hinrichs, K.U., Hehemann, J.H., 2020. Laminarin is a major molecule in the marine carbon cycle. *Proceedings of the National Academy of Sciences* 117, 6599–6607.
- Cai, C.M., Zhang, T., Kumar, R., Wyman, C.E., 2014. Integrated furfural production as a renewable fuel and chemical platform from lignocellulosic biomass. *Journal of Chemical Technology & Biotechnology* 89, 2–10.
- Engel, A., Händel, N., 2011. A novel protocol for determining the concentration and composition of sugars in particulate and in high molecular weight dissolved organic matter (hmw-dom) in seawater. *Marine Chemistry* 127, 180–191.
- Engel, A., Thoms, S., Riebesell, U., Rochelle-Newall, E., Zondervan, I., 2004. Polysaccharide aggregation as a potential sink of marine dissolved organic carbon. *Nature* 428, 929–932.
- Fang, W., Xue, S., Deng, P., Zhang, X., Wang, X., Xiao, Y., Fang, Z., 2019. AmyZ1: a novel α -amylase from marine bacterium *Pontibacillus* sp. ZY with high activity toward raw starches. *Biotechnol Biofuels* 12, 95.

567 Field, C.B., Behrenfeld, M.J., Randerson, J.T., Falkowski, P., 1998. Primary
568 production of the biosphere: integrating terrestrial and oceanic components.
569 science 281, 237–240.

570 Guillard, R., 1983. Culture of phytoplankton for feeding invertebrate animals.
571 Culture of marine invertebrates , 108–132.

572 Guillard, R., Hargraves, P., 1993. *Stichochrysis immobilis* is a diatom, not a
573 chrysophyte. Phycologia 32, 234–236.

574 Harrison, P.J., Waters, R.E., Taylor, F., 1980. A broad spectrum artificial sea wa-
575 ter medium for coastal and open ocean phytoplankton 1. Journal of phycology
576 16, 28–35.

577 Hedges, J.I., Baldock, J.A., Glinas, Y., Lee, C., Peterson, M., Wakeham, S.G.,
578 2001. Evidence for non-selective preservation of organic matter in sinking
579 marine particles. Nature 409, 801–804.

580 Imberty, A., Bulon, A., Tran, V., Prez, S., 1991. Recent advances in knowl-
581 edge of starch structure. Starch-Strke 43, 375–384.

582 Kappelmann, L., Krger, K., Hehemann, J.H., Harder, J., Markert, S., Un-
583 fried, F., Becher, D., Shapiro, N., Schweder, T., Amann, R.L., et al., 2019.
584 Polysaccharide utilization loci of north sea flavobacteriia as basis for using
585 susc/d-protein expression for predicting major phytoplankton glycans. The
586 ISME journal 13, 76–91.

587 Kobayashi, T., Tanabe, I., Obayashi, A., 1974. On the properties of the starch
588 granules from unicellular green algae. Agricultural and Biological Chemistry
589 38, 941–946.

- 590 Laine, R.A., 1994. A calculation of all possible oligosaccharide isomers both
591 branched and linear yields 1.05×10^{12} structures for a reducing hexasaccha-
592 ride: the isomer barrier to development of single-method saccharide sequencing
593 or synthesis systems. *Glycobiology* 4, 759.
- 594 Lever, M., 1972. A new reaction for colorimetric determination of carbohydrates.
595 *Analytical biochemistry* 47, 273–9.
- 596 McCleary, B., Solah, V., Gibson, T., 1994. Quantitative measurement of total
597 starch in cereal flours and products. *Journal of Cereal Science* 20, 51–58.
- 598 McCleary, B.V., McNally, M., Rossiter, P., 2002. Measurement of resistant starch
599 by enzymatic digestion in starch and selected plant materials: collaborative
600 study. *Journal of AOAC International* 85, 1103–1111.
- 601 Megazyme, 2018. Amylose/amylopectin. Amylose/Amylopectin Assay Procedure
602 for the Measurement of the Amylose and Amylopectin Contents of Starch
603 06/18, 1–10.
- 604 Myklestad, S., 1974. Production of carbohydrates by marine planktonic diatoms.
605 i. comparison of nine different species in culture. *Journal of Experimental*
606 *Marine Biology and Ecology* 15, 261–274.
- 607 Nelson, C.E., Attia, M.A., Rogowski, A., Morland, C., Brumer, H., Gardner, J.G.,
608 2017. Comprehensive functional characterization of the glycoside hydrolase
609 family 3 enzymes from *cellvibrio japonicus* reveals unique metabolic roles in
610 biomass saccharification. *Environmental microbiology* 19, 5025–5039.
- 611 Painter, T.J., 1983. Algal polysaccharides, in: *The polysaccharides*. Elsevier, pp.
612 195–285.

613 Panagiotopoulos, C., Sempéré, R., 2005. Analytical methods for the determina-
614 tion of sugars in marine samples: A historical perspective and future directions.
615 *Limnology and Oceanography: Methods* 3, 419–454.

616 Ral, J.P., Derelle, E., Ferraz, C., Wattebled, F., Farinas, B., Corellou, F., Buléon,
617 A., Slomianny, M.C., Delvalle, D., d'Hulst, C., et al., 2004. Starch division
618 and partitioning. a mechanism for granule propagation and maintenance in
619 the picophytoplanktonic green alga *ostreococcus tauri*. *Plant Physiology* 136,
620 3333–3340.

621 Sheath, R.G., Hellebust, J.A., Sawa, T., 1979. Floridean starch metabolism
622 of *porphyridium purpureum* (rhodophyta) i. changes during ageing of batch
623 culture. *Phycologia* 18, 149–163.

624 Sorokina, O., Corellou, F., Dauvillée, D., Sorokin, A., Goryanin, I., Ball, S.,
625 Bouget, F.Y., Millar, A.J., 2011. Microarray data can predict diurnal changes
626 of starch content in the picoalga *ostreococcus*. *BMC systems biology* 5, 36.

627 Suzuki, E., Suzuki, R., 2013. Variation of storage polysaccharides in phototrophic
628 microorganisms. *Journal of applied glycoscience* 60, 21–27.

629 Unfried, F., Becker, S., Robb, C.S., Hehemann, J.H., Markert, S., Heiden, S.E.,
630 Hinzke, T., Becher, D., Reintjes, G., Krüger, K., et al., 2018. Adaptive mech-
631 anisms that provide competitive advantages to marine bacteroidetes during
632 microalgal blooms. *The ISME journal* 12, 2894.

633 Wong, P.Y., Lai, Y.H., Puspanadan, S., Ramli, R.N., Lim, V., Lee, C.K., 2019.
634 Extraction of starch from marine microalgae, *chlorella salina*: Efficiency and
635 recovery. *International Journal of Environmental Research* 13, 283–293.

636 Yu, S., Blennow, A., Bojko, M., Madsen, F., Olsen, C.E., Engelsen, S.B., 2002.
637 Physico-chemical characterization of floridean starch of red algae. Starch-
638 Stärke 54, 66–74.