

1 **Characterization of spirolide producing *Alexandrium ostenfeldii***
2 **(Dinophyceae) from the western Arctic**

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13 **ABSTRACT**

14 Toxin producing dinoflagellates of the genus *Alexandrium* Halim represent a risk to
15 Arctic environments and economies. This study provides the first record and a
16 characterization of *Alexandrium ostenfeldii* in the western Arctic. During a cruise along the
17 coasts of western and southern Greenland 36 isolates of the species were established in
18 August 2012. Plankton samples taken at 3 different stations from the upper water layer at
19 water temperatures of approx. 4-7 °C, contained low amounts of *A. ostenfeldii*. Sequencing of
20 SSU and ITS-LSU rDNA and subsequent phylogenetic analyses identified all Greenland
21 strains as members of a NW Atlantic spirolide producing phylogenetic clade. Molecular
22 results were confirmed by morphological features typical for this group (= Group 5 of a
23 recent ITS-LSU phylogeny of *A. ostenfeldii*). The Greenland isolates did not contain either
24 Paralytic Shellfish Poisoning toxins or gymnodimines, but produced several spirolides.
25 Altogether 12 different analogues were detected, of which only SPX-1, C, 20-meG and H
26 have been described earlier. The remaining 8 spirolides have not been identified so far. Some
27 of them were found to dominate the toxin profiles of a number of isolates. Among the 36
28 investigated strains spirolide composition varied considerably, particularly isolates from
29 western Greenland (Station 516) exhibited a high diversity of analogues, with different
30 profiles in nearly all 22 isolates. All of the 34 tested Greenland strains showed considerable
31 lytic capacity when exposed to *Rhodomonas salina*.

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33 Key-words: *Alexandrium ostenfeldii*, Greenland, spirolides, lytic activity

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36 1. INTRODUCTION

37

38 World-wide, the majority of the toxic bloom-forming harmful algal species belong to the
39 dinoflagellate genus *Alexandrium*. **Species of *Alexandrium*** are often globally distributed,
40 occurring in a variety of habitats and all geographic zones (Taylor et al., 1995; Lilly et al.,
41 2007). Many *Alexandrium* species are able to produce potent toxins, such as paralytic
42 shellfish toxins (PSTs), which affect the neuromuscular, sensory, digestive and
43 cardiovascular systems of human and other vertebrates (Hallegraeff, 1993; Selina et al.,
44 2006) and account for most of the harmful events caused by members of the genus (Anderson
45 et al., 2012). These algal toxins represent a serious risk for the environment and human health
46 (Hallegraeff, 1993).

47 One of the less studied toxic species of the genus is *Alexandrium ostenfeldii*. It has
48 been widely observed in temperate waters of Europe (Balech and Tangen, 1985), North
49 America (Cembella et al., 2000a), the Russian Arctic (Okolodkov and Dodge, 1996) and
50 Eastern Siberian Seas (Konovalova, 1991). There are also records of the occurrence of *A.*
51 *ostenfeldii* from the coast of Spain (Fraga and Sanchez, 1985), the Mediterranean (Balech,
52 1995), New Zealand (Mackenzie et al., 1996), Peru (Sánchez et al., 2004) and Japan (Nagai
53 et al., 2010). However, for a long time, *A. ostenfeldii* has been considered mainly as a
54 background species, occurring at low cell concentrations mixed with other bloom forming
55 dinoflagellates (Balech and Tangen, 1985; Moestrup and Hansen, 1988; John et al., 2003).
56 Only in the past decade it has gained increasing attention when dense blooms of this species
57 (or its synonym *A. peruvianum*) were reported e.g. from South America (Sánchez et al.,
58 2004), the Northern Baltic Sea (Kremp et al., 2009), along the Adriatic coast of Italy
59 (Ciminiello et al., 2006), the estuaries of the US East coast (Tomas et al., 2012), and, most
60 recently, the Netherlands (Burson et al., 2014). It is not clear whether the recent increase in

61 bloom events is due to anthropogenic spreading or changing environmental conditions
62 favoring bloom formation. Most of the recent blooms occurred during summer in coastal
63 areas and were associated with warm water periods (e.g. Hakanen et al., 2012). Experimental
64 studies indicate that increased water temperature has a favorable effect on *A. ostenfeldii*
65 bloom populations and it has been suggested that changing climate conditions promote bloom
66 formation (Kremp et al., 2012). The species produces PSP toxins (Hansen et al., 1992),
67 spirolides (Cembella et al., 2000a) and gymnodimines (Van Wagoner et al., 2011), and all
68 compounds may even occur together in one strain (Tomas et al., 2012). Thus, an increase of
69 *A. ostenfeldii* bloom events with several potent toxins involved may represent a new risk to
70 the environment that is associated with climate change.

71 Most of the global *A. ostenfeldii* records are from cold-water environments and the
72 species has long been considered to have an arctic-boreal distribution (Okolodkov, 2005). It
73 was originally described from the north-east coast of Iceland (Paulsen, 1904), and has
74 thereafter been reported mainly from high latitude waters of the North Atlantic (Cembella et
75 al., 2000a; Brown et al., 2010), Scandinavia (Tangen, 1983; Moestrup and Hansen, 1988) as
76 well as arctic and subarctic waters of northern Siberia and the Russian Far East (Konovalova,
77 1991; Okolodkov, 2005; Selina et al., 2006). In a recent study on *A. tamarensis* in Greenland,
78 the presence of *A. ostenfeldii* in the western Arctic was briefly mentioned (Baggesen et al.,
79 2012).

80 It has been predicted that anthropogenic climate change is causing dramatic changes the
81 Arctic area, including increased temperature (Screen and Simmonds, 2012) and rapid decline
82 of glaciers, ice cover (Comiso et al., 2008), ice thickness (Kwok and Rothrock, 2009), and
83 resulting in ice-free summer conditions in future. These changes will have large effects on
84 many marine species including primary producers (Wassmann et al., 2008). Though the
85 responses of the Arctic marine ecosystems to climate change are not well known, temperature

86 increase has been considered one of the changes affecting the performance, abundance and
87 distribution of arctic organisms most significantly (Alcaraz et al., 2014). Temperature
88 increase and larger ice-free regions have, for example, been suggested to expand the
89 distribution ranges of HAB-species into or within the Arctic sea-area (Hallegraeff, 2010) and
90 cause severe problems to the sensitive Arctic environment due to toxin production, and their
91 accumulation in higher trophic levels.

92 Since *A. ostenfeldii* is present in arctic and subarctic waters, it could be one of the first
93 harmful dinoflagellate species to be favored by the increase of water temperature and the
94 predicted cascading effects of climate change in the ecosystem (Walsh et al., 2011).
95 Alternatively, populations from temperate coastal waters of the North Atlantic or Pacific area
96 may expand their ranges and cause toxic blooms in the Arctic. Most of the recently reported
97 *A. ostenfeldii* blooms are caused by representatives of a brackish, warm-water adapted
98 globally distributed genotype (Tomas et al., 2012; Kremp et al., 2014). They differ from most
99 of the other *A. ostenfeldii* isolates by their potential to produce PSP toxins in addition to or
100 instead of spirolides and to potentially produce neurotoxic gymnodimines. This genotype has
101 recently expanded within the northern Baltic Sea, a boreal cold-water system, presumably as
102 result of increased summer surface temperatures (Kremp et al., 2009), and now regularly
103 forms toxic blooms here. Most North Atlantic isolates, including subarctic strains from
104 northern Iceland, though cluster in a different phylogenetic group and mainly produce
105 spirolides. Spirolides are potent neurotoxins causing rapid death of mice when injected
106 intraperitoneally and are thus regarded as “emerging” toxins, even if the currently are not
107 regarded as toxic to humans and therefore not regulated.

108 Despite abundant records of *A. ostenfeldii* from arctic coasts, arctic populations have not
109 been characterized in terms of phylogenetic affiliation and important phenotypic traits such
110 as morphology, toxicity and allelopathic potency. Such information is important for assessing

111 the potential for bloom formation and risks of toxicity in a region where shellfish industry is
112 an important part of the local economy (Garcia, 2006). Here we present molecular,
113 morphological and physiological data of multiple *A. ostenfeldii* strains isolated from western
114 and southern Greenland and provide the first, to our knowledge, extensive phylogenetic and
115 morphological characterization as well as a detailed description of toxin profiles and lytic
116 capacity of arctic populations of this species.

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118

119 **2. MATERIAL AND METHODS**

120

121 **2.1 Sampling and sample preparation**

122 A total of 36 clonal strains of *Alexandrium ostenfeldii* were established from water
123 samples collected at three stations at the west coast of Greenland (Fig. 1) during a cruise
124 aboard the research vessel “Maria S. Merian” in August 2012. Vertical net tows were
125 conducted at each station through the upper 30 m of the water column with a 20- μ m-mesh
126 Nitex plankton net. Total volume of each net tow concentrate was measured and a 20 ml
127 subsample was fixed with paraformaldehyde (1% final concentration).

128 Seawater samples were taken at standard depths of 3, 8, and 20 m depth by means of 5 L
129 Niskin entrapment bottles mounted on a remotely triggered rosette-sampler. 50 mL water
130 samples were fixed with neutral Lugol (2 % final concentration) in brown glass bottles.

131

132 **2.2 Plankton composition**

133 For a qualitative and quantitative characterization of the plankton community at the three
134 stations where *A. ostenfeldii* were isolated, both net tow and bottle samples were inspected
135 microscopically. For net tow concentrates, 0.5 mL of the PFA-fixed samples (corresponding

136 to 0.1 % of the entire net tow) was counted in small sedimentation chambers. From lugol-
137 fixed Niskin bottle samples, 10 mL each for all three depths per station were settled in 10 mL
138 settling chambers. Depending on the size and/or abundance of different categories these were
139 counted in the whole chamber or in representative sub-areas. All counts were performed
140 using an inverted microscope (Zeiss Axiovert 40C).

141

142 **2.3 Cultures**

143 Single cells of *Alexandrium* were isolated onboard from live net tow concentrates under
144 a stereomicroscope (M5A, Wild, Heerbrugg, Switzerland) by micropipette. Single cells were
145 transferred into individual wells of 96-well tissue culture plates (TPP, Trasadingen,
146 Switzerland) containing 250 μL of K medium (Keller et al., 1987) prepared from 0.2 μm
147 sterile-filtered natural Antarctic seawater diluted with seawater from the sampling location at
148 a ratio of 1:10. Plates were incubated at 10°C in a controlled environment growth chamber
149 (Model MIR 252, Sanyo Biomedical, Wood Dale, USA). After 3 to 4 weeks, unialgal isolates
150 were transferred to 24-well tissue culture plates, each well containing 2 mL of K medium
151 diluted 1:5 with Antarctic seawater. Exponentially growing isolates were finally used as
152 inoculum for batch cultures in 65 mL polystyrene cell culture flasks and were maintained
153 thereafter at 10° C under a photon flux density of 30-50 $\mu\text{mol m}^{-2} \text{s}^{-1}$ on a 16:8 h light:dark
154 photocycle in a temperature-controlled walk-in growth chamber. Different sets of cultures
155 were maintained in K-medium and f/2 –Si enriched (Guillard and Ryther, 1962) sterilized
156 filtered Baltic sea water adjusted to a salinity of 35. For all strains, species designation was
157 confirmed by fluorescence microscopy of calcofluor-stained samples. All strains were
158 analysed for toxins (PSP, spirolides and gymnodimines); lytic capacity was estimated for all
159 but two strains which were lost before these analyses could be performed. Detailed
160 morphometric analysis and molecular data were generated for 7 and 14 selected strains,

161 respectively (Table 1).

162

163 **2.4 DNA extraction and Phylogenetic analyses**

164 To determine the phylogenetic position of 14 selected Greenland strains and their ITS,
165 D1-D2 LSU and SSU rDNA sequences, cells were harvested from exponentially growing
166 cultures and their DNA was extracted and processed to sequencing as explained in detail in
167 Kremp et al. (2014). For ITS1 through D1-D2 LSU phylogenetic analysis, we used 14
168 sequences from Greenland together with 32 additional *A. ostenfeldii* strains obtained from
169 Genbank (Table S1) together with sequences of closely related *A. minutum* and *A. insuetum*.
170 For the SSU alignment (1684 bp) we used one strain from each sampling station and 67 SSU-
171 sequences of *A. ostenfeldii* and other *Alexandrium* species generated in this study or obtained
172 from Genbank (Table S1). The ITS-LSU sequences (1246 bp) and SSU sequences (1684 bp)
173 were aligned using MAFFT (Multiple Alignment with Fast Fourier Transform) (Kato et al.,
174 2009), with default settings, as implemented in SeaView (Gouy et al., 2010). The resulting
175 alignments were deposited in a public web server (“PopSet” at ENTREZ), and will be
176 provided upon request.

177 Bayesian inferences (BI) were performed using the software MrBayes v3.2 (Ronquist
178 and Huelsenbeck, 2003) with the GTR+G substitution model (Rodriguez et al., 1990),
179 selected under the Bayesian Information Criterion (BIC) with jModelTest 0.1.1. (Posada,
180 2008). For priors, we assumed no prior knowledge on the data. Two runs of four chains (three
181 heated and one cold) were executed for 10×6^{15} generations, sampling every 500 trees. In each
182 run, the first 25% of samples were discarded as the burn-in phase. The stability of model
183 parameters and the convergence of the two runs were confirmed using Tracer v1.5 (Rambaut
184 and Drummond, 2007). Additionally, separate maximum likelihood phylogenetic trees based
185 on either ITS-LSU or SSU alignments were calculated in GARLI 2.0 (Zwickl, 2006) with

186 parameters estimated from the data, using an evolutionary model GTR+G, selected under the
187 Akaike Information Criterion (AIC) with jModelTest 0.1.1. (Posada, 2008). Tree topology
188 was supported with bootstrap values calculated with 1000 replicates.

189

190 **2.5 Morphological characterization**

191 For all strains, species designation was confirmed by fluorescence microscopy of
192 calcofluor-stained samples. For a more detailed morphometric characterization of the
193 Greenland isolates, 7 strains representing the 3 sampling stations were randomly chosen. For
194 those, cell size measurements and plate observations were performed using light and
195 epifluorescence microscopy. Cells were collected from exponentially growing cultures and
196 preserved with 1-2% neutral Lugol's solution. To determine cell length and width, fixed cells
197 were viewed under a Leica DMI3000B inverted microscope (Leica, Wetzlar, Germany) and
198 photographed at 400x magnification with a Leica DFC 490 digital camera. Measurements
199 were taken using the analysis tool of LAS (Leica Application Suite) camera software.

200 Distinctive thecal plates were visualized under epifluorescence after applying a few drops of
201 a 1 mg L⁻¹ solution of Fluorescent Brightener 28 (Sigma-Aldrich). Evaluation of plate shapes
202 (1' and s.a. plate) and plate measurements (1', s.a. and 6'') were carried out on images of
203 cells photographed at 630 x magnification.

204

205 **2.6 Toxin analyses**

206 For toxin analysis, strains were grown in 65 mL plastic culture flasks at the standard
207 culture conditions described above. For each harvest, cell density was determined by settling
208 lugol fixed samples and counting >600 cells under an inverted microscope. Cultures at a cell
209 density ranging from ranging from 400 - 5.000 cells mL⁻¹ were harvested by centrifugation
210 (Eppendorf 5810R, Hamburg, Germany) at 3220 g for 10 min, 50 mL for analyzing PSP

211 toxins and 15 mL for analysis of cyclic imines. Cell pellets were transferred to 1 mL
212 microtubes, again centrifuged (Eppendorf 5415, 16,000 g, 5 min), and stored frozen (-20°C)
213 until use.

214 Cyclic imine toxins including spiroptides and gymnodimines (GYMs) were analyzed by
215 liquid chromatography coupled to tandem mass spectrometry (MS^2). Mass spectral
216 experiments were performed on an ABI-SCIEX-4000 Q Trap (Applied Biosystems,
217 Darmstadt, Germany), equipped with a TurboSpray[®] interface coupled to an Agilent
218 (Waldbronn, Germany) model 1100 LC. The LC equipment included a solvent reservoir, in-
219 line degasser (G1379A), binary pump (G1311A), refrigerated autosampler
220 (G1329A/G1330B), and temperature-controlled column oven (G1316A).

221 After injection of 5 μL of sample, separation of spiroptides was performed by reversed-
222 phase chromatography on a C8 column (50×2 mm) packed with 3 μm Hypersil BDS 120 \AA
223 (Phenomenex, Aschaffenburg, Germany) and maintained at 25°C . The flow rate was 0.2 mL
224 min^{-1} and gradient elution was performed with two eluents, where eluent A was water and
225 eluent B was methanol/water (95:5 v/v), both containing 2.0 mM ammonium formate and 50
226 mM formic acid. Initial conditions were elution with 5% B, followed by a linear gradient to
227 100% B within 10 min and isocratic elution until 10 min with 100% B. The programme was
228 then returned to initial conditions within 1 min followed by 9 min column equilibration (total
229 run time: 30 min).

230 Mass spectrometric parameters were as follows: curtain gas: 20 psi, CAD gas: medium,
231 ion spray voltage: 5500 V, temperature: 650°C , nebulizer gas: 40 psi, auxiliary gas: 70 psi,
232 interface heater: on, declustering potential: 121 V, entrance potential: 10 V, exit potential: 22
233 V, collision energy: 57 V. Selected reaction monitoring (SRM) experiments were carried out
234 in positive ion mode by selecting the transitions shown in Table 2. Dwell times of 40 ms
235 were used for each transition.

236 Paralytic shellfish poisoning (PSP) toxins were analyzed by liquid chromatography with
237 post-column derivatization and fluorescence detection as described in Suikkanen et al.
238 (2012). Limits of quantification ($s/n = 5$) for the individual PSTs on column were as follows:
239 GTX4: 1190 pg, GTX1: 1570 pg, GTX2: 63 pg, GTX3: 67 pg, STX: 61 pg, NEO: 585 pg,
240 B1: 329 pg.

241

242

243 **2.7 Estimation of lytic capacity**

244 Isolates were screened for lytic activity by using a *Rhodomonas* bioassay (Tillmann et
245 al., 2009). Clonal isolates of *A. ostenfeldii* were grown in batch cultures in 65 mL plastic
246 culture flasks at standard culture conditions described above and were regularly inspected
247 with a stereomicroscope. When cultures became dense (2.000-7.000 cells mL⁻¹) cell
248 concentration of each strain was estimated by counting Lugol's iodine fixed cells within a
249 subsample that contained at least 600 cells. Cultures were subsequently diluted with medium
250 to a final cell concentration of approximately 1.000 cells mL⁻¹. Then 3.9 mL of diluted
251 cultures was dispensed into triplicate 6 mL glass vials. Two negative and one positive control
252 (triplicate each) were performed in the same way as the experimental assays. The first
253 negative control contained only K medium (3.9 mL), whereas the second negative control
254 was performed with *A. tamarense*, strain Alex5, a strain which previously was shown to be
255 non-lytic (Tillmann and Hansen, 2009). The positive control was performed by adding 3.9
256 mL of a culture of the allelochemically active *A. tamarense* strain Alex 2 (Tillmann and
257 Hansen, 2009). Each sample was spiked with 0.1 mL of a *Rhodomonas* culture which was
258 adjusted (based on microscope cell counts) to 4×10^5 cells mL⁻¹ yielding a final start
259 concentration of 1×10^4 mL⁻¹ of the target cells in the bioassay. Samples were then incubated
260 for 24 h in the dark at 10°C. Subsequently, samples were fixed with 2% Lugol's iodine

261 solution and concentration of intact target cells was determined. All counts were performed
262 with an inverted microscope (Zeiss Axiovert 40C, Göttingen, Germany) in small counting
263 chambers with a volume set up for cell counts of 0.5 mL. A sub-area of the chamber
264 corresponding to at least 600 *Rhodomonas* cells in the control was counted. In order to
265 quantify lytic effects, only intact cells of the target species were scored. Strains of *A.*
266 *ostenfeldii* were simultaneously tested in groups of 3-10 strains in a total of 4 bioassay runs.
267 All results were expressed as final concentration of *Rhodomonas* expressed as percent of the
268 seawater control.

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270

271 3. RESULTS

272

273 3.1 Plankton situation

274 We successfully isolated *Alexandrium ostenfeldii* from three stations located on the west
275 coast of Greenland (Fig. 1). At the northernmost station 506 located in the Uummannaq
276 Fjord, surface water of rather high temperature (5-7 °C) and low salinities (27-28) was
277 layered above cold (2 °C) and saline (32.7) water at 30 m depth. In contrast, at station 516
278 located south of Disco Island in the Disco Bay, salinity was constantly high (33.2-33.5) in the
279 upper 30 m. Surface temperature here was 6.8 °C in the upper 10 m and decreased steadily to
280 2.5 °C in 30 m. The lowest surface temperature was recorded at the southernmost station 524
281 (3.7 °C in the first 15 m, decreasing to 2.7 °C at 30 m depth) with salinity in the upper 30 m
282 ranging from 30.6 to 31.2.

283 Phytoplankton density at all three stations was generally low with chlorophyll values
284 ranging from 0.26 (Stat. 506, 30 m) to 2.0 µg L⁻¹ (Stat 516, 20 m) with highest values at
285 depth of about 20 m (Daniela Voß, per. comm.). Plankton communities at the three stations

286 might be characterised as representing a post spring-bloom situation with relatively low
287 biomass of photosynthesizing organisms and with a large and diverse proportion of
288 heterotrophs (e.g tintinnids, aloricate ciliates, rotatoria, heterotrophic dinoflagellates).

289 At the northernmost station 506 there were some remains of the diatom bloom present,
290 mainly species of *Thalassiosira*, large amounts of an unidentified small (<10 µm) diatom and
291 some larger chain forming *Fragillaria* species. The most abundant species was the
292 mixotrophic colony-forming chrysophyte *Dinobryon* sp. with up to 764 cells mL⁻¹.
293 Photosynthetic dinoflagellets identified in net or Niskin samples included *Dinophysis* spp.,
294 *Scrippsiella* sp. *Protoceratium reticulatum*, and *Gonyaulax* spp. A quite diverse assemblage
295 of heterotrophic dinoflagellates including various species of the genus *Protoberidinium* was
296 present.

297 The net tow sample of Station 516 was quite dilute and characterised by a variety of
298 different dinoflagellate species with just a few diatom cells (*Cerataulina bergonii*,
299 *Thalassiosira nordenskiöldii*, *Leptocylindrus* sp.) present. Most abundant in Niskin bottle
300 samples were *Scrippsiella* sp. (17-30 mL⁻¹, range of three depth samples), *Protoberidinium*
301 spp. (25-32 mL⁻¹) and ciliates (11-30 mL⁻¹), and unidentified small and medium-sized (10-30
302 µm) dinoflagellates (47-83 mL⁻¹). With densities up to 1216 mL⁻¹ *Dinobryon* sp. was even
303 more abundant compared to station 506.

304 Station 524 located at the southernmost tip of Greenland was distinctly different. The
305 chrysophyte *Dinobryon* completely disappeared and the plankton was dominated by large
306 amount of diatoms of the genus *Pseudonitzschia* (86-107 mL⁻¹) accompanied by some
307 *Thalassiosira*. Most of the larger dinoflagellates were of the genus *Protoberidinium* with a
308 few cells of *Dinophysis* spp., *Ceratium arcticum*, *C. fusus*, *Gonyaulax* spp. and
309 *Protoceratium reticulatum* present.

310 The density of *Alexandrium* spp. at the three stations was generally low. Based on Niskin
311 bottle samples, *Alexandrium* spp. (not determined at the species level) ranged between 0 and
312 a maximum of 500 cells L⁻¹ recorded at Station 516 (15 meter). Quantification of
313 *Alexandrium* spp. in net tow samples indicated an abundance of 87 x10³ (Stat 506) to 750 x
314 10³ (Stat 524) cells per square meter in the upper 30 meter of the water column. At station
315 516 *A. ostenfeldii* co-occurred with *A. tamarensense* and *A. tamutum*, as all three species were
316 successfully isolated from the same sample (to be reported elsewhere). In contrast, all
317 *Alexandrium*-like cells isolated from station 524 and successfully brought into culture turned
318 out to be *A. ostenfeldii*. Many of the *Alexandrium* cells observed in net tow sample from
319 station 524 contained large inclusion (Fig. 2).

320

321 **3.2 Phylogenetic position of Greenland isolates**

322 All of the selected 14 strains from the 3 sampling stations had identical ITS, LSU and
323 SSU sequences. Bayesian Inference (BI) and Maximum Likelihood (ML) methods returned
324 identical tree topologies for ITS-LSU data set (Fig. 3). In the phylogenetic tree, Greenland
325 *Alexandrium* strains from all 3 stations grouped together with each other and with strains
326 from Iceland and the Gulf of Maine (USA), constituting a well-supported monophyletic (ML
327 99 %, BI 1.0) clade, consistent with group 5 defined by Kremp et al. (2014). BI and ML
328 analyses of SSU sequences (supplementary figure S1) showed a different, more conserved
329 tree topology, where *A. ostenfeldii* was not grouped into 6 different groups as based on ITS
330 and LSU sequences, but into three major groups. The first group collates groups 1 and 2 of
331 the ITS-LSU phylogeny, placing strains from the Baltic Sea, US East coast estuaries and
332 China in the same cluster with isolates from the UK, Ireland and Spain (ML 90 %, BI 0.93).
333 The second group (ML 82 %, BI 0.99) is identical with group 6 of the ITS-LSU phylogeny
334 and a third group (ML 55 %, BI 0.56) combines ITS-LSU groups 3, 4 and 5. Here again, the

335 Greenland isolates are most similar to strains from the NW Atlantic, and appear slightly
336 differentiated from the Japanese (ML 82 %, BI 100) and New Zealand populations (ML 83
337 %, BI 100).

338

339 **3.3 Morphology**

340 Cells of *Alexandrium ostenfeldii* from Greenland were round to ellipsoid in shape (Fig. 4
341 A, B). Mean cell size varied among the examined strains with largest cells (mean cell length
342 = 45.78 ± 6.47) found in strain P1H10 and smallest cells found in P1D5 ($28.83 (\pm 2.41)$). Most
343 strains were of medium size with mean cell lengths of 33 to 37 μm (Table 3). With mean
344 width to height ratios of 0.89- 0.97, the majority of the examined strains were slightly longer
345 than wide (Table 3). Most round cells were found in strain P1F8, while cells were particularly
346 elongated in strain P2G3.

347 Dimensions of plates varied among strains, largest 1' plates were found in the largest
348 cells (strain P1H10) (Table 3). Most of the examined strains had narrow 1' plates (Fig. 4 B,
349 C), with the angular shape and the large ventral pore typical for *A. ostenfeldii*. The right
350 anterior margins of cells from Greenland strains were mostly straight with a few cases where
351 curved or irregular margins were detected (Table 3, Fig. 4 C, E). Two of the 7 analyzed
352 strains, P1H10 and P2G3 had a significant amount of cells whose 1' plate was anteriorly
353 extended. Except for strain P2G3, which contained a significant amount of cells with A-
354 shaped s.a. plates, the vast majority of examined cells from the investigated strains had door-
355 latch-shaped s.a. plates (Table 3, Fig. 4 B, D, E). Commonly, a fold was observed on these
356 plates (Fig. 4 E). Width to height ratios of the anterior sulcal plate (s.a.) revealed that these
357 plates were generally lower than high (Table 3). This was also the case for the 6'' plate
358 (Table 3, Fig. 4 E). Generally the variability in w/h measurements of the s.a. and 6'' plates
359 was high as indicated by high standard deviations. The pore plate with the comma-shaped

360 apical pore (Fig. 4 G) and the sulcal plates (Fig. 4 H) showed the typical shapes and
361 arrangements of *Alexandrium ostenfeldii*.

362

363 **3.4 Toxin composition**

364 All the 36 analyzed strains were spirolide producers, but none of them produced
365 gymnodinime A (GYM-A), 12-methyl GYM-A or paralytic shellfish poisoning (PSP) toxins.
366 The limits of detection (LODs) for PSP toxins expressed as cell quotas are quite variable
367 depending on the different sensitivities of individual PSP toxins and the varying amounts of
368 cells used for analysis (depending on the growth of individual strains). The lowest LOD was
369 0.005 pg cell⁻¹ of the most sensitive GTX-2 for strain P3F1 with most harvested cells and the
370 highest LOD was 8.4 pg cell⁻¹ of the least sensitive GTX-1 for strain P2F3 with the lowest
371 number of harvested cells.

372 Spirolide compositions among strains were very diverse (Table 4), however, the most
373 frequent spirolides were spirolide C and 20-methyl spirolide G. Even though no standards for
374 spirolide C and 20-methyl spirolide G are available, their product ion spectra are well
375 documented in the literature (Hu et al., 2001; Aasen et al., 2005) and they could be identified
376 by the comparison of collision induced dissociation (CID) product ion spectra. In addition,
377 spirolide H (Roach et al., 2009) was also identified by CID spectra comparison. The fourth
378 spirolide unambiguously identified was 13-desmethyl spirolide C, which is the only spirolide
379 for which a standard is commercially available. Besides these four spirolides, there were
380 eight other compounds with CID spectra characteristic for spirolides, i.e. the cyclic imine
381 fragments m/z 150, 164 or 180 and the formation of the “F1” fragment (Sleno et al., 2004),
382 which is formed by a retro-Diels-Alder reaction and the cleavage of a C₁₅-element including
383 the lactone moiety. These putative spirolides could not unambiguously be assigned or they
384 have not been reported in the literature yet. For molecular masses and CID spectra see

385 supplementary material. Spirolide profiles of strains isolated from station 524 were similar
386 and spirolides C and 20-methyl G in all strains from this station made up more than 90% of
387 total spirolides. In contrast, spirolide composition and abundances were more diverse among
388 strains isolated from station 516 (Tab. 4). Spirolide cell quotas ranged from very low levels
389 of 0.02 pg cell⁻¹ up to 66 pg cell⁻¹ (data not shown).

390

391 **3.5 Lytic activity**

392 Screening for lytic capacity performed at one cell density of approximately 1000 cells
393 (685-1300, mean = 941, SD = 115) indicates that with one exception (see below), all strains
394 of *A. ostenfeldii* at that concentration clearly had the capacity to lyse the target *Rhodomonas*
395 *salina* (Fig. 5). In all bioassay runs, positive controls (using the known lytic *A. tamarensis*
396 strain Alex2) yielded total lysis of *Rhodomonas*, whereas in all negative controls using the
397 non-lytic strain Alex5 were not significantly different from seawater controls (data not shown).
398 At the fixed dose of ca. 1000 cells mL⁻¹, lytic capacity varied considerably with the final
399 number of intact *Rhodomonas* ranging from 0 to 92%. When tested with a simple t-test, final
400 *Rhodomonas* concentration incubated with strain P1G6 was not significantly different to the
401 control. An additional test of strain P1G6 tested at a distinctly higher dose (ca. 3000 cells mL⁻¹)
402 clearly showed that this strain is lytic as well (result not shown).

403

404

405 **4. DISCUSSION**

406

407 Recent phylogenetic investigations of the *Alexandrium ostenfeldii* species complex
408 (including *A. peruvianum*) revealed that global isolates are genetically differentiated into 6
409 groups (Kremp et al. 2014). In the respective concatenated ITS-LSU phylogeny, these groups

410 fall into two major clusters, one consisting groups 1 and 2 which contain a mix of geographic
411 isolates from shallow estuarine, often brackish habitats, and the other one containing
412 geographically differentiated Groups 3 to 6. The ITS-LSU analysis performed in the present
413 study reproduced the Kremp et al. (2014) phylogeny and identified the Greenland isolates as
414 members of Group 5, representing *A. ostenfeldii* populations from the western coasts of the
415 North Atlantic. Also in the SSU phylogeny, Group 5 strains, including the representative
416 Greenland isolates grouped together, however, with this more conserved marker, the groups
417 were not as well resolved. Group 5 strains are nested in a cluster together with Group 3 and 4
418 isolates from New Zealand and Japan. SSU analysis emphasize the close relationship of
419 Group 1 and 2 strains suggested by morphological and physiological similarities found earlier
420 (Kremp et al., 2014): the two groups appear collated when compared with the more
421 conserved SSU marker (suppl. Figure S1).

422 The morphological characters found in the Greenland isolates are consistent with their
423 molecular identity and placement in Group 5. The typically longer than wide cells from the
424 Greenland material mostly exhibited narrow 1' plates, door-latch shaped s.a. plates and low
425 6'' plates, features which are most commonly found in the closest genetic and geographic
426 neighbors from the Gulf of Maine, Atlantic Canada and Northern Iceland (Kremp et al.,
427 2014). Cell dimensions, plate shapes and w/h measurements of the s.a. and 6'' plates varied
428 somewhat among the studied strains, as typical for *A. ostenfeldii*. Mean cell length was
429 generally smaller than reported from field material (Balech and Tangen, 1985; Gribble et al.,
430 2005) but cell size measurements of the Greenland strains were on average comparable to
431 other cultured Group 5 isolates (Kremp et al., 2014). It has been suggested earlier that
432 cultured cells of *A. ostenfeldii* are generally smaller than in their natural environment (John et
433 al., 2003).

434 Though the geographic distance between the Greenland population and other Group 5
435 isolates is considerable, the Greenland strains very likely represent a natural extension of this
436 group into the western Subarctic and Arctic. *A. ostenfeldii* has been reported from the
437 northern St Lawrence Estuary (Levasseur et al., 1998). Sequence data is not available from
438 this location, but likely these occurrences extend the Gulf of Maine and Nova Scotia
439 populations. Being present in the northern Gulf of St Lawrence which opens to the North
440 Atlantic, *A. ostenfeldii* is exposed here to the Subpolar Gyre, which connects the eastern coast
441 of North America with the coasts of Greenland and Iceland. Group 5 representatives
442 generally seem to thrive in marine cold-water environments: A study on the Gulf of Maine
443 and Nova Scotia isolates showed that growth rates were higher at 10 degrees than at 15
444 degrees (Cembella et al., 2000b; Cembella et al., 2000a). Gribble et al. (2005) found that the
445 numbers of *A. ostenfeldii* cells decreased in the water column as water temperatures increased
446 in late spring. Generally, *A. ostenfeldii* is widely distributed in cold water environments such
447 as the Russian Arctic (Okolodkov, 2005), and often reported from spring phytoplankton
448 communities (Paulsen, 1904; Balech and Tangen, 1985; Moestrup and Hansen, 1988;
449 Levasseur et al., 1998) emphasizing that a cold-water ecotype of this species commonly
450 occurs. The observations of cold water occurrences of *A. ostenfeldii* in the Gulf of Maine,
451 Atlantic Canada and Iceland (Paulsen, 1904), suggests that Group 5 represents this cold water
452 ecotype. In the present study we do not systematically address the ecological preferences of
453 the Greenland isolates, but it can be noted that most isolates grow better when maintained at
454 11 °C compared to 16 °C (J. Oja, personal communication) suggesting that they are adapted
455 to cooler rather than warmer water.

456 Large toxic blooms have so far mostly been related with Group 1 genotypes. Recently,
457 blooms of this genotype have been expanding in brackish US east coast estuaries, river
458 estuaries of Western Europe and in the Baltic Sea (Hakanen et al., 2012; Tomas et al., 2012;

459 Burson et al., 2014). Because of the clustering of the Greenland isolates in cold-water
460 adapted phylogenetic Group 5, a comparable temperature related expansion of *A. ostenfeldii*
461 is not expected in Greenland. Group 1 differs physiologically from Group 5 by being adapted
462 to low salinities and thriving in warm water. In contrast to Group 5, Group 1 *A. ostenfeldii*
463 produces paralytic shellfish toxins (PST's) in addition to or instead of spirolides and
464 gymnodimines and blooms are of concern because they are associated with high PST
465 concentrations in the water (Burson et al., 2014).

466 Consistent with the Group 5 isolates investigated earlier, only spirolides were detected in
467 the 36 Greenland isolates analyzed here for toxin composition. Particularly *A. ostenfeldii*
468 from Groups 2, 4 and 5 seem to lack the ability to produce PSTs and gymnodimines due to
469 complete or partial absence of the respective genes (Suikkanen et al., 2013). A particularly
470 striking feature of the Greenland isolates is the high diversity of spirolide analogues. To date
471 14 different spirolides are known (Molgó et al., 2014), but spirolide diversity in *A. ostenfeldii*
472 seems to be higher, as the Greenland isolates apparently produce at least 8 spirolides not
473 reported in the literature. This lack of knowledge has two reasons; 1) spirolides do not belong
474 to regulated shellfish toxins and accordingly there is no economically driven interest in
475 research into this field and 2) spirolides are large molecules with many options for slight
476 modifications such as hydroxylation, hydration/dehydration or methylation, which may and
477 apparently do result in many analogs of the same structural body. These modifications may
478 be introduced by slight modifications of the synthesizing enzymes over evolutionary times and
479 is observed in other toxin classes as well.

480 The few available studies on spirolide composition also suggest that spirolide variability
481 in *A. ostenfeldii* is generally high. On one hand already 14 different spirolides haven been
482 comprehensively described and structurally elucidated from strains of different geographic
483 locations (Molgó et al., 2014). On the other, Gribble et al. (2005) detected up to 7 different

484 spirolides and found high spirolide variation among 15 strains of *A. ostenfeldii* from the Bay
485 of Fundy, North West Atlantic, a geographically very constrained area. Our findings (12
486 different spirolides in 36 strains from 3 stations) confirm this pattern. The CID spectrum of
487 compound 2 (Fig. S2F) is consistent with spirolide A, but due to lack of any reference
488 material an unambiguous identification is not possible. Compound 1 (Fig. S2E) has a 14 Da
489 smaller molecular ion than compound 2 and thus may be a yet unreported desmethyl spirolide
490 A. Interestingly there were three compounds with the molecular mass of m/z 722 present.
491 (Figs. S2J, K, and L). Compound 6 instead of the commonly observed cyclic imine fragments
492 of m/z 150 or 164 showed a fragment of m/z 180, which for the first time was described for
493 27-hydroxy-13-desmethyl spirolide C by Ciminiello et al. (2010). The CID spectrum of
494 compound 6 with a 14 Da higher molecular mass than 27-hydroxy-13-desmethyl spirolide C
495 is consistent with 27-hydroxy spirolide C; however, these are only hypothetical structures
496 which have to be confirmed by NMR. In addition there are spirolides with unusual molecular
497 masses such as compound 4 (m/z 696) and compound 5 (m/z 720) (Figs. S2H and I). The fact
498 that of the 12 spirolides detected in the Greenland isolates described here, 8 are yet
499 undescribed or at least not unambiguously attributed to known spirolides, highlights the need
500 for further research in this field

501 We estimated the cell quota of total spirolide content to be ranging from very low levels
502 of $0.02 \text{ pg cell}^{-1}$ up to 66 pg cell^{-1} . Although cultures were grown under identical
503 environmental conditions, strains considerably differed (although not quantified) in growth
504 performance and cell yield. Cultures were thus not harvested at the same growth stages
505 and/or cell density and this may have partly influenced spirolide cell quota. Cell quota for
506 one strain of *A. ostenfeldii* from Canada has been described to vary almost ten-fold depending
507 on environmental condition, ranging from ca. 30 to 240 pg cell^{-1} (Maclean et al., 2003) For
508 20 of the 36 Greenland strains we estimated a cell quota of less than 1 pg per cell which is

509 rather low compared to these literature values. Cell quota of field samples have been shown
510 to be quite variable as well ranging from 168 pg per cell to no detectable spirolides despite
511 rather high concentrations of *A. ostenfeldii* (Gribble et al., 2005). Cell quotas of 1 to 60 pg
512 per cell estimated for 16 Greenland strains are well in the range of other studies where cell
513 quotas of about 6 to 66 pg per cell are reported (Cembella et al., 2000a; Gribble et al., 2005;
514 Tatters et al., 2012).

515 In addition to the production of spirolide toxins, all strains of *A. ostenfeldii* from
516 Greenland produce alleochemicals with the capacity to lyse cells of the target species
517 *Rhodomonas*. Lytic activity of extracellular secondary metabolites is rather widespread in the
518 genus *Alexandrium* and has been shown to affect other microalgae (Arzul et al., 1999;
519 Tillmann et al., 2008), heterotrophic protists (Hansen et al., 1992; Matsuoka et al., 2000;
520 Tillmann and John, 2002) and microbial communities (Weissbach et al., 2011). Deleterious
521 effects in particular of *A. ostenfeldii* on other microorganisms have been known for a long
522 time. Hansen et al. (1992) described cell lysis of tintinnid predators of a Danish isolate of *A.*
523 *ostenfeldii* in culture experiments, which they – at that time – discussed as potentially related
524 to the PSP toxin content of that *A. ostenfeldii* strain. Although molecular structures and exact
525 mode of action of alleochemicals from *Alexandrium* still are poorly known (Ma et al., 2009;
526 Ma et al., 2011) it is now clear that they are unrelated to the known toxins produced by this
527 genus (Tillmann and John, 2002; Tillmann et al., 2007). In the latter paper, three strains of *A.*
528 *ostenfeldii* from different geographic origin and with or without spirolides all showed
529 deleterious effects on a number of protistan species. There are indications that isolates of *A.*
530 *ostenfeldii* from other areas are lytic as well: haemolytic activity has been described for
531 *Alexandrium peruvianum* (= *A. ostenfeldii*) from coastal waters of North Carolina (Tatters et
532 al., 2012; Tomas et al., 2012) and production of alleochemicals has been shown for isolates
533 of *A. ostenfeldii* from the Baltic Sea which deter copepod grazers by unknown chemical

534 substances (Sopanen et al., 2011), and negatively affect co-occurring phytoplankton
535 (Hakanen et al., 2014).

536 We used a simple one-concentration bioassay to show lytic activity and we do not yet
537 have full dose-response curves that are needed to estimate EC₅₀ (cell concentration of *A.*
538 *ostenfeldii* causing lysis of 50% of the *Rhodomonas* population) values. Nevertheless, our
539 data show that EC₅₀ values of most Greenland isolates grown at 10 °C seem to be well below
540 1000 cells mL⁻¹ and would thus be in the range of EC₅₀ value estimated for temperate
541 isolates, which have been shown to range from 0.3 to 1.9 x 10³ cells mL⁻¹ (Tillmann et al.,
542 2007). This is in the range of values determined for Baltic isolates (Hakanen et al., 2014),
543 suggesting similar lytic capacities in the different phylogenetic groups of *A. ostenfeldii*. EC₅₀
544 values for hemolysis of *A. ostenfeldii* from the US coast given by Tomas et al. (2012) and
545 Tatters et al. (2012) for the same strain, seem to be orders of magnitude higher but refer to
546 different target cells and procedures than used in standard assays.

547 Our screening also indicates that there are profound quantitative differences in lytic
548 activity between different isolates. It has to be kept in mind that our strains were grown at
549 exactly the same environmental conditions but have not been sampled at a defined growth
550 stage, which might have contributed to the observed strain differences. Quantitative
551 differences in lytic activity within a population of *Alexandrium* have been described before
552 for isolates of *A. tamarense* from the northern North Sea (Alpermann et al., 2010). Such a
553 high phenotypic variability, also manifested here in the high variability in the spirolide
554 profile with *A. ostenfeldii* from Greenland, and manifested in the North Sea population of *A.*
555 *tamarense* by a high strain variability in PSP toxin profile, has been discussed as evidence for
556 lack of strong selective pressure on respective phenotypic traits at the time the population
557 was sampled (Alpermann et al., 2010).

558 Lytic effects at cell concentrations used in this study were almost three orders of
559 magnitude above the densities of *Alexandrium* spp. estimated in the field samples during our
560 field expedition. Nevertheless, motile phytoplankton, such as *Alexandrium* spp, may
561 accumulate in horizontal layers under certain conditions, along thermoclines or the water
562 surface (MacIntyre et al., 1997; Mouritsen and Richardson, 2003) and the resulting high
563 densities may be accompanied by effective concentrations of secondary metabolites in these
564 layers

565 Lytic compounds produced by *A. ostenfeldii* may be involved in cell-to-cell interactions,
566 e.g. in prey capture. A number of allelochemically active microalgae, including species of
567 *Alexandrium* and *A. ostenfeldii*, have been shown to be mixotrophic (Jacobson and Anderson,
568 1986; Tillmann, 1998; Jeong et al., 2005; Stoecker et al., 2006; Yoo et al., 2009; Sheng et al.,
569 2010; Blossom et al., 2012) and it has been speculated that allelochemicals are used for
570 predation. Large food vacuoles, as observed here for most of the specimen at station 524
571 (Fig. 2) have been described for *A. ostenfeldii* for a number of field sample sites (Jacobson
572 and Anderson, 1986; Gribble et al., 2005). In our experiments, however, we did not observe
573 any particulate uptake of *Rhodomonas* by *A. ostenfeldii* and clearly more detailed
574 experiments are needed to clarify mixotrophy in *A. ostenfeldii* and a potential role of lytic
575 activity in prey capture.

576 To conclude, spirolide producing and lytic *A. ostenfeldii* are present along the west coast
577 of Greenland. In accordance with the phylogenetic analysis, the arctic cold water population,
578 however, does not produce PSP toxins and thus does not contribute to the PSP toxicity in the
579 region (Baggesen et al., 2012) which is thus probably caused exclusively by *A. tamarense*.
580 Spirolides currently are not considered dangerous to humans at the concentrations found in
581 shellfish and are therefore not regulated, but they clearly are potent neurotoxins causing rapid
582 death of mice when injected intraperitoneally. Furthermore, they were found to be toxic to

583 mice in oral feeding studies, and are therefore regarded as so-called “emerging” toxins. Our
584 results show the presence of numerous new spirolide analogs whose specific toxicity
585 currently is unknown. Low cell concentrations of *A. ostenfeldii* as found in plankton samples
586 during our summer cruise and the preference for cold water where slow growing
587 dinoflagellates are usually outcompeted by fast growing diatoms, does not exclude the
588 possibility that this species may, under certain circumstances, form blooms. An increase of
589 dinoflagellate proportions and dinoflagellate dominated blooms has been reported from other
590 cold-water systems (Klais et al., 2011). In the Baltic Sea, the recent increase of dinoflagellate
591 spring blooms has been related to favourable effects of changing climate conditions on the
592 recruitment of the respective species from their cyst beds, which provides them a competitive
593 advantage over diatoms (Kremp et al., 2008; Klais et al., 2011). Also *A. ostenfeldii* forms
594 resting cysts (Mackenzie et al., 1996) and hence the seasonal dynamics may largely depend
595 on cyst germination and formation processes that are potentially influenced by changing
596 environmental conditions. In fact, cysts of *Alexandrium* have been detected in West
597 Greenland sediments (Mindy Richlen, pers. com). It is not known whether life cycle
598 regulated indirect effects of bloom promotion could eventually also favour cold-water *A.*
599 *ostenfeldii* in coastal waters of western Greenland. Further field studies and ecophysiological
600 experiments targeting the life cycle, growth performance and toxin production at different
601 environmental conditions are now needed to estimate the impact of global change and
602 temperature increase on the survival, establishment, extension, and bloom formation of
603 *Alexandrium* spp. and to fully evaluate the risk potential of algal toxins for arctic regions with
604 shellfish industry as an important and rising part of the local economy.

605

606

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867

868 **FIGURE CAPTIONS**

869

870 **Figure 1.** Map of the southern part of Greenland with sampling stations at the western and
871 southern coasts.

872

873 **Figure 2.** Light micrographs of *A. ostenfeldii* cells from field samples collected at station 524
874 containing food vacuoles. A and B representing two different focal planes of the same cell.

875 Scale bars = 15 μ m

876

877 **Figure 3.** Bayesian tree derived from a concatenated ITS2-5.8-ITS2-D1/D2 LSU alignment.

878 Node labels correspond to posterior probabilities from Bayesian inference and bootstrap
879 values from maximum likelihood, ML, analyses (ML/BI).

880

881 **Figure 4.** Cell morphology. A and B: Light micrographs of a life cell of strain P2G3 in

882 ventral view at different focus. Arrows on A point to the ends of the U-shaped nucleus,

883 arrows on B emphasize visible 1' (first apical) plate with ventral pore and s.a. (anterior

884 sulcal) plate. C-H: epifluorescence micrographs of cells of strain P1G3 stained with

885 fluorescent brightener: (C) 1' and (D) doorlatch-shaped s.a. plate (arrow), (E) cell in ventral

886 view, (F) lateral plates, (G) apical plates and apical pore (arrow), and (H) sulcal plates. s.d.a.:

887 right anterior lateral sulcal plate, s.s.a.: left anterior lateral sulcal plate, s.p.: posterior sulcal

888 plate. Scale bars: A- B = 15 μ m, C,D,H = 5 μ m, E = 20 μ m; F, G = 10 μ m.

889

890 **Figure 5.** Lytic activity of 34 *A. ostenfeldii* isolates from western and southern Greenland.

891 Stations represented by different colors: white bars = Station 506, grey bars = Station 516 and

892 black bars = Station 524.

893

894 TABLES

895

896 Table 1: Information on analyses performed on *Alexandrium ostenfeldii* isolates from

897 Greenland

898

Strain	Station	Morphological species confirmation	Detailed morphometric analysis	Sequences	PSP toxins	Spirolides	Lyse test
P1 D5	506	x	x	SSU, ITS, LSU	x	x	x
P1 H10	516	x	x	SSU, ITS, LSU	x	x	x
P2 E3	516	x		SSU, ITS, LSU	x	x	x
P2 E4	516	x		SSU, ITS, LSU	x	x	x
P2 F2	516	x		SSU, ITS, LSU	x	x	x
P2 F3	516	x			x	x	x
P2 F4	516	x			x	x	x
P2 F7	516	x			x	x	
P2 G2	516	x			x	x	x
P2 G9	516	x		SSU, ITS, LSU	x	x	x
P2 H4	516	x			x	x	x
P2 H8	516	x			x	x	x
P3 F1	516	x	x	SSU, ITS, LSU	x	x	x
P4 C6	516	x			x	x	x
P4 E3	516	x			x	x	x
P4 D8	516	x			x	x	
P4 F10	516	x			x	x	x
P4 G2	516	x			x	x	x
P3 A12	516	x			x	x	x
P2 H2	516	x			x	x	x
P2 G3	516	x	x	SSU, ITS, LSU	x	x	x
P3 E4	516	x		SSU, ITS, LSU	x	x	x
P4 F4	516	x			x	x	x
P1 F5	524	x	x	SSU, ITS, LSU	x	x	x
P1 F7	524	x			x	x	x
P1 F8	524	x	x	SSU, ITS, LSU	x	x	x
P1 F9	524	x		SSU, ITS, LSU	x	x	x
P1 F10	524	x			x	x	x
P1 F11	524	x			x	x	x
P1 G3	524	x	x	SSU, ITS, LSU	x	x	x
P1 G5	524	x			x	x	x
P1 G11	524	x			x	x	x
P1 G8	524	x			x	x	x
P1 F6	524	x			x	x	x
P1 F4	524	x			x	x	x
P1 G6	524	x		SSU, ITS, LSU	x	x	x

899

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901

902

903 Table 2: Mass transitions m/z (Q1>Q3 mass) and their respective cyclic imine toxins.

904

Mass transition	toxin	Collision energy (CE) [V]
508>490	GYM-A	57
522>504	12-me GYM -A	57
640>164	undescribed	57
644>164	undescribed	57
650>164	H	57
658>164	undescribed	57
674>164	undescribed	57
678>164	13,19-didesme C	57
678>150	undescribed	57
692>164	13-desme C, G, undescribed	57
692>150	A, undescribed	57
694>164	13-desme D, undescribed, pinnatoxin G	57
694>150	B	57
696>164	undescribed	57
698>164	undescribed	57
706>164	C, 20-me G	57
708>164	D	57
710>164	undescribed	57
710>150	undescribed	57
720>164	undescribed	57
722>164	undescribed	57
766>164	pinnatoxin F	57
784>164	pinnatoxin E	57

905

Table 3: Cell dimensions and plate morphometry in representative strains from the three sampling stations.

strain	Cell size		Cell Ratio w/h	N	Plate morphometry						
	Cell width	Cell length			1' % straight margin	1' % Ext.	1' area (μm^2)	s.a.% Door-latch	s.a. Ratio w/h	6'' Ratio w/h	N
Station 506											
P1 D5	27.78 (± 1.92)	28.83 (± 2.41)	0.97 (± 0.05)	25	80	0	36.19 (± 4.60)	87	1.49 (± 0.20)	1.17 (± 0.22)	15
Station 516											
P1 H10	43.65 (± 6.38)	45.78 (± 6.47)	0.95 (± 0.04)	25	90	20	99.50 (± 18.76)	70	1.54 (± 0.28)	1.23 (± 0.10)	10
P3 F1	31.45 (± 2.30)	33.10 (± 2.71)	0.95 (± 0.03)	25	87	7	60.76 (± 13.41)	100	1.70 (± 0.20)	1.32 (± 0.13)	15
P2 G3	30.70 (± 1.85)	34.53 (± 2.72)	0.89 (± 0.05)	25	100	47	72.43 (± 16.29)	53	1.57 (± 0.30)	1.34 (± 0.17)	15
Station 524											
P1 F5	31.31 (± 2.59)	34.09 (± 3.88)	0.92 (± 0.07)	25	73	0	58.69 (± 10.74)	93	1.51 (± 0.20)	1.34 (± 0.14)	15
P1 F8	35.73 (± 3.18)	37.10 (± 4.07)	0.97 (± 0.13)	25	80	0	68.93 (± 16.03)	93	1.56 (± 0.21)	1.36 (± 0.14)	15
P1 G3	30.12 (± 2.99)	33.19 (± 2.62)	0.91 (± 0.06)	25	93	7	73.56 (± 16.20)	93	1.48 (± 0.16)	1.36 (± 0.13)	15

Table 4. Percent distribution of spiroside analogues. Numbers in bold represent relative abundances > 1%. SPX-1 = 13-desmethyl spiroside C; C = spiroside C; 20-me G = 20-methyl spiroside G; H = spiroside H; Cp = compound

	Stat	SPX-1	C	20-meG	H	Cp 1	Cp 2	Cp 3	Cp 4	Cp 5	Cp 6	Cp 7	Cp 8
P1 D5	506	-	77.1	16.8	-	-	-	-	-	-	3.4	2.7	-
P1 H10	516	0.7	-	84.3	-	0.1	-	0.3	-	0.8	-	-	13.7
P2 E3	516	31.2	-	-	41.3	-	27.5	-	-	-	-	-	-
P2 E4	516	19.2	-	-	-	1.2	-	-	-	70.1	-	-	9.4
P2 F2	516	5.1	63.6	-	6.7	0.1	4.1	-	-	16.6	-	3.8	-
P2 F3	516	-	82.9	17.1	-	-	-	-	-	-	-	-	-
P2 F4	516	2.7	57.3	39.7	-	-	-	-	-	-	0.1	-	-
P2 F7	516	1.4	31.1	-	25.1	1.2	10.0	-	-	29.0	-	-	2.3
P2 G2	516	0.2	40.2	18.4	7.1	-	11.1	-	-	11.6	-	-	11.3
P2 G9	516	-	100.0	-	-	-	-	-	-	-	-	-	-
P2 H4	516	-	95.4	4.6	-	-	-	-	-	-	-	-	-
P2 H8	516	2.4	-	89.0	-	-	-	-	-	0.3	-	-	8.2
P3 F1	516	0.2	-	81.7	-	-	0.1	0.2	-	1.0	-	-	16.9
P4 C6	516	-	50.3	49.7	-	-	-	-	-	-	-	-	-
P4 E3	516	1.2	96.3	2.2	-	-	-	0.3	-	-	-	-	-
P4 D8	516	-	31.4	-	14.1	-	-	9.0	36.1	9.4	-	-	-
P4 F10	516	-	68.6	20.0	0.2	-	-	-	-	-	-	-	11.3
P4 G2	516	-	-	100.0	-	-	-	-	-	-	-	-	-
P3 A12	516	-	52.2	-	47.8	-	-	-	-	-	-	-	-
P2 H2	516	-	-	92.5	-	-	-	0.9	-	-	-	-	6.6
P2 G3	516	18.1	1.3	0.1	-	-	-	-	-	72.7	-	-	7.8
P3 E4	516	0.2	99.6	-	-	-	-	0.2	-	-	-	-	-
P4 F4	516	-	77.4	19.6	0.3	-	-	0.4	-	2.1	-	-	-
P1 F5	524	0.1	79.5	19.8	-	-	-	0.2	-	-	0.1	0.2	-
P1 F7	524	0.1	78.3	20.9	-	-	0.1	0.4	-	-	0.1	-	-
P1 F8	524	-	92.1	6.6	-	-	0.1	0.4	-	-	0.2	0.5	-
P1 F9	524	0.2	64.7	33.2	-	-	0.1	1.4	-	-	0.3	-	-
P1 F10	524	0.1	68.4	30.5	-	-	0.1	0.9	-	-	0.1	-	-
P1 F11	524	-	87.3	6.4	-	-	-	-	-	-	-	5.4	-
P1 G3	524	0.6	92.3	6.6	-	-	-	0.6	-	-	-	-	-
P1 G5	524	0.7	76.1	21.7	0.4	-	-	0.4	-	-	0.3	0.4	-
P1 G11	524	0.2	84.2	14.2	0.5	-	-	0.3	-	-	0.3	0.3	-
P1 G8	524	-	88.6	6.5	-	-	-	-	-	-	-	4.9	-
P1 F6	524	0.1	85.1	13.8	-	-	0.1	0.1	-	-	0.1	0.3	-
P1 F4	524	0.1	77.2	22.3	-	-	-	-	-	-	0.1	0.2	-
P1 G6	524	-	70.5	28.0	0.3	-	-	0.6	-	-	0.2	0.3	-

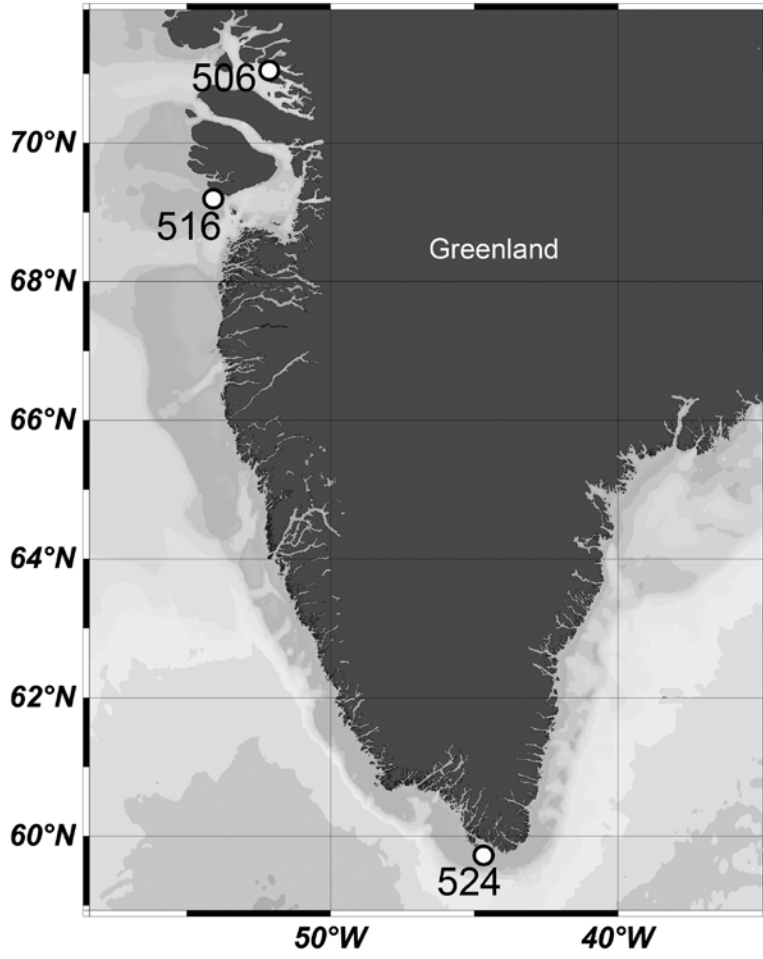


Fig. 1

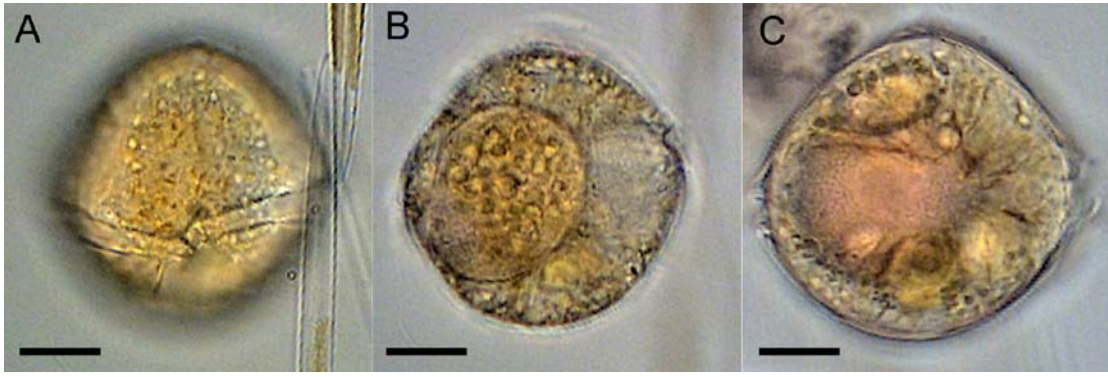


Fig. 2

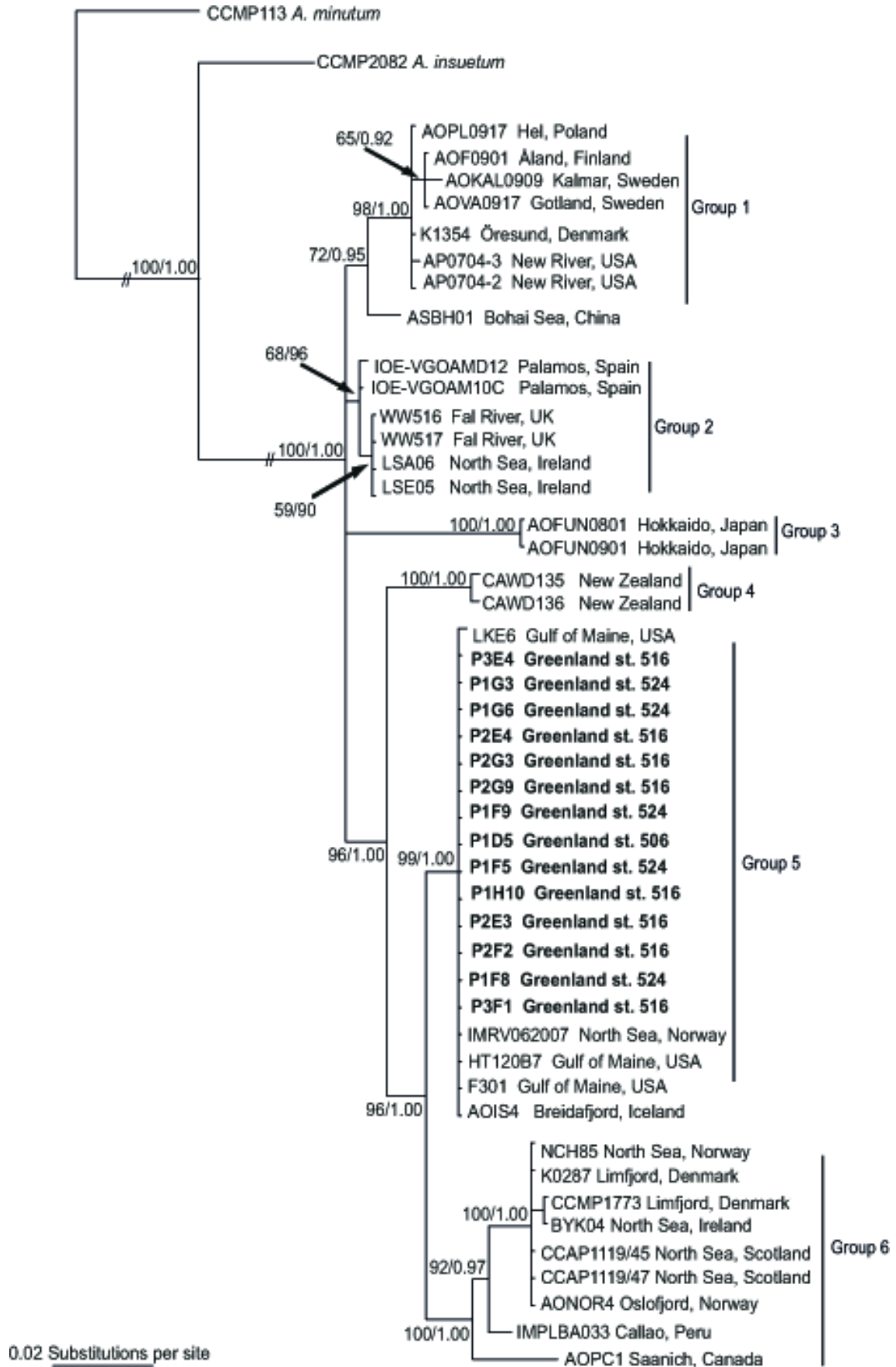


Fig. 3

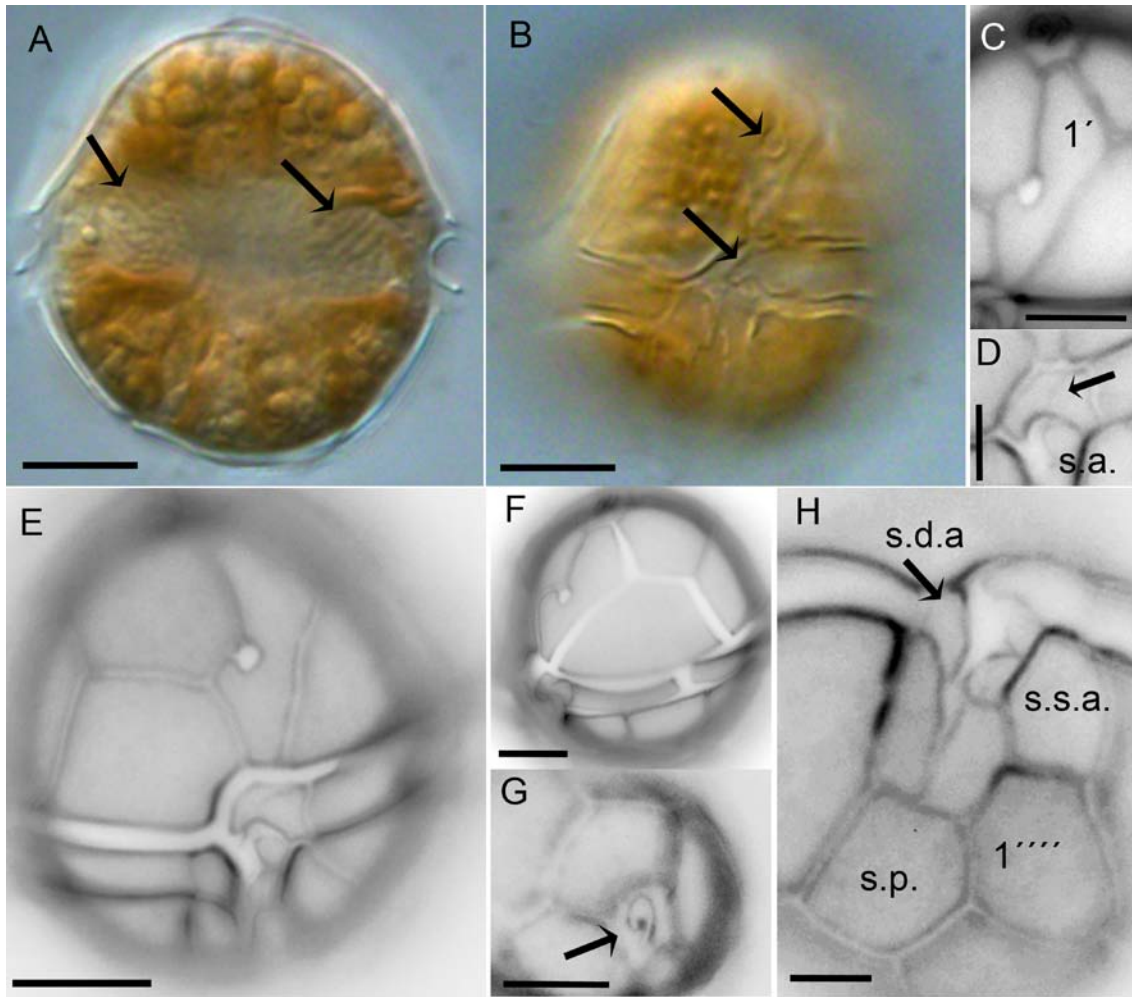


Fig. 4

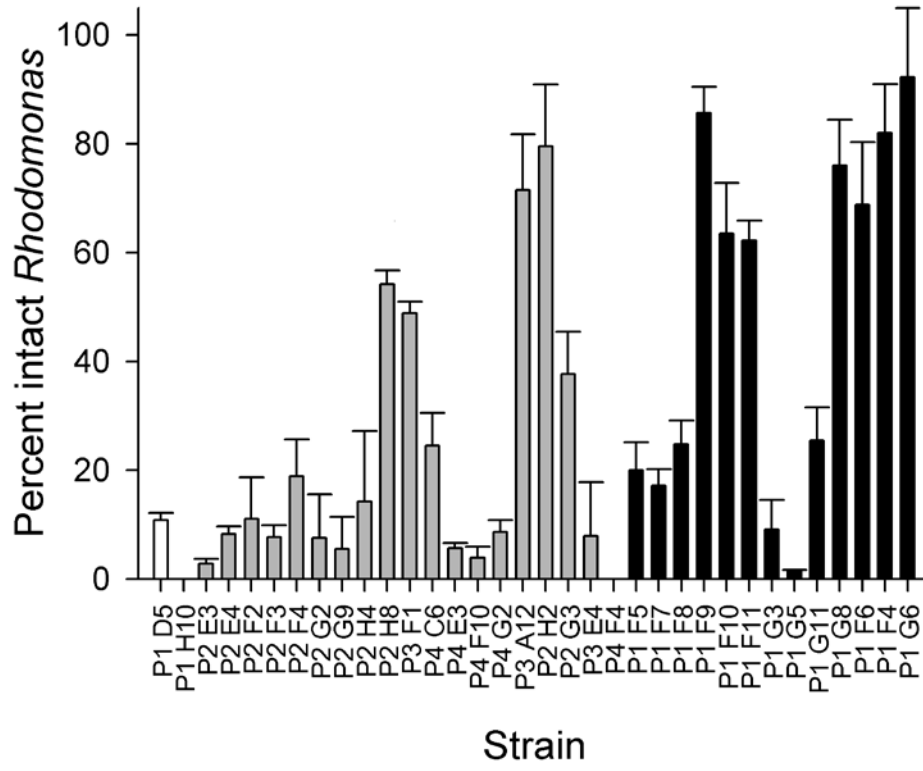


Fig. 5