DIFFERENCES IN THE CHEMICAL COMPOSITION OF ORGANIC-WALLED DINOFLAGELLATE RESTING CYSTS FROM PHOTOTROPHIC AND HETEROTROPHIC DINOFLAGELLATES[1]

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Condensed running title

Dinoflagellate cyst compositional differences

ABSTRACT

Dinoflagellates constitute a large proportion of the planktonic biomass from marine to freshwater environments. Some species produce a preservable organic-walled resting cyst (dinocyst) during the sexual phase of their life cycle that is an important link between the organisms, the environment in which their parent motile theca grew, and the sedimentary record. Despite their abundance and widespread usage as proxy indicators for environmental conditions, there is a lack of knowledge regarding the dinocyst wall chemical composition. It is likely that numerous factors, including phylogeny and life strategy, determine the cyst wall chemistry. However, the extent to which this composition varies based on inherent (phylogenetic) or variable (ecological) factors has not been studied.

To address this, we used micro-Fourier transform infrared (FTIR) spectroscopy to analyze nine cyst species produced by either phototrophic or heterotrophic dinoflagellates from the extant orders Gonyaulacales, Gymnodiniales and Peridiniales. Based on the presence of characteristic functional groups, two significantly different cyst wall compositions are observed that correspond to the dinoflagellate's nutritional strategy. The dinocyst wall compositions analyzed appeared carbohydrate-based, but the cyst wall produced by phototrophic dinoflagellates suggested a cellulose-like glucan, while heterotrophic forms produced a nitrogen-rich glycan. This constitutes the first empirical evidence nutritional strategy is related to different dinocyst wall chemistries. Our results indicated phylogeny was less important for predicting composition than the nutritional strategy of the dinoflagellate, suggesting potential for cyst wall chemistry to infer past nutritional strategies of extinct taxa preserved in the sedimentary record.

Keywords

dinoflagellate cyst, dinosporin, heterotrophic, infrared spectroscopy, macromolecule, nutritional strategy, phototrophic

Abbreviations

FTIR, Fourier transform infrared

DCM, dichloromethane

EtOH, ethanol

Pyr-GC-MS, pyrolysis gas chromatography-mass spectrometry

INTRODUCTION

Dinoflagellates are biflagellate, eukaryotic protists that comprise a large proportion of planktonic biomass (Taylor 1987) and are therefore important components of marine and freshwater ecosystems. Some species, as part of their sexual reproduction and in preparation for a dormant period in their life cycle, produce an organic-walled, refractory resting cyst (Wall and Dale 1968, Pfiester and Anderson 1987, Head 1996) capable of being preserved in the sediment record. The existence of two life cycle stages has resulted in the creation of two taxonomic systems: one used by biologists and based on the morphology of living motile cells, and the other developed by paleontologists and based on resting cyst morphology. Dinoflagellate resting cysts (dinocysts) are important sources of environmental information for modern marine (e.g., Rochon et al. 1999, Dale et al. 2002, Matthiessen et al. 2005, Holzwarth et al. 2007, Pospelova et al. 2008, Bouimetarhan et al. 2009, Holzwarth et al. 2010, Zonneveld et al. 2013, Bringué et al. 2013) and freshwater (e.g., Kouli et al. 2001, Tardio et al. 2006, Leroy et al. 2009, McCarthy et al. 2011, Mertens et al. 2012) ecosystems, and are also valuable for the reconstruction of oceanographic conditions (e.g., Reichart and Brinkhuis 2003, Pospelova et al. 2006, González et al. 2008). They therefore represent the link between organisms, the environment, and the sedimentary record. However, dinocyst taxa react in different ways to oxidation; cysts from the order Peridiniales show high sensitivity, while cysts from the order Gonyaulacales demonstrate greater resistance, both in laboratory and natural settings (Dale 1976, Zonneveld et al. 1997, 2001, Combourieu-Nebout et al. 1998, McCarthy et al. 2000, Hopkins and McCarthy 2002, Mertens et al. 2009a). This suggests that intrinsic properties of the dinocyst walls vary between the different orders, some physical, such as differences in wall thickness or structure, while others are chemical. In this study, we focused on potential differences in cyst wall chemistry.

To date, little is known regarding the macromolecular composition of dinocyst walls, which is comprised of a refractory biomacromolecule referred to as dinosporin (Fensome et

al. 1993). Different compositions have been suggested, including mainly aromatic (Kokinos et al. 1998), a mixture of aromatic and aliphatic (Hemsley et al. 1994), and mainly aliphatic and heavily cross-linked (Versteegh et al. 2007). More recently, a carbohydrate-based dinosporin has been suggested on the basis of Fourier transform infrared (FTIR) analyses and pyrolyses of Lingulodinium machaerophorum (Deflandre and Cookson) Wall from culture and surface sediments (Versteegh et al. 2012). Further micro-FTIR spectroscopic analyses of late Paleocene dinocysts in the genus *Apectodinium* (Costa and Downie) Lentin and Williams agreed with this interpretation but also displayed considerable differences in composition between species of the same genus (Bogus et al. 2012). Therefore, it seems that phylogeny could be a factor contributing to dinosporin compositional differences. However, there are also other factors with significant potential to influence dinosporin composition, including differences in the compounds available within the motile dinoflagellate cell which builds the cyst, and differences resulting from post-depositional alteration. It is assumed that dinoflagellates build their cysts from material readily available within the cell (Kokinos and Anderson 1995, Hallet, 1999), which was suggested by TEM studies of cyst formation in the freshwater species *Ceratium hirundinella* (Müller) Dujardin (Chapman et al. 1982). Furthermore, there is clear evidence that dinocyst morphology can vary with differing environmental conditions (e.g., Hallet 1999, Ellegaard 2000, Lewis et al. 2001, Ellegaard et al. 2002, Zonneveld and Susek 2007, Mertens et al. 2009b) as well as evidence that some dinoflagellates, such as *Karlodinium veneficum* (Ballantine) Larsen and *Alexandrium minutum* Halim (Fuentes-Grünewald et al. 2009, 2012) can adapt their cellular composition to different environmental conditions. However, it is not known whether there is a link between chemical compounds available within the motile cell and dinosporin composition. One way to investigate potential impacts on dinosporin chemistry is to analyze dinocysts produced by dinoflagellates that have very different life strategies, such as nutritional strategies (phototrophy versus heterotrophy). Additionally, diagenetic processes, such as natural

vulcanization, have been shown to chemically alter the cyst walls of Thalassiphora pelagica (Eisenack) Benedek and Gocht emend. (Versteegh et al. 2007) and raised the question as to what extent the differences observed between Apectodinium species were influenced by differential diagenesis (Bogus et al. 2012). However, we circumvented the diagenetic factor by utilizing specimens isolated from modern marine and lacustrine surface sediments where the exposure to diagenetic processes is short and thus expected to be small.

We investigated differences in the dinocyst wall chemistry in species from different lineages (the orders Gonyaulacales, Gymnodiniales, and Peridiniales). The main objectives were to determine the primary factors that affect dinosporin composition and to address the extent to which a particular dinocyst composition was monophyletic, i.e., different dinoflagellate lineages produce different wall compositions, or polyphyletic and subject to variations resulting from different nutritional strategies, since these are known to be polyphyletic. The dinocysts were analyzed using micro-FTIR spectroscopy. This versatile technique has already been successfully used to identify compounds in the complex biopolymers of dinocyst wall layers of optically identified and individually isolated specimens (e.g., Kokinos et al. 1998, Versteegh et al. 2007, 2012, Bogus et al. 2012).

MATERIAL AND METHODS

Sample treatment

Samples were retrieved from marine and lacustrine locations (Fig. 1, Table 1). Surface sediment samples from the marine Benguela upwelling region (GeoB 2341, 4804), off the coast of northwest Africa (GeoB 6010), and freshwater Honey Harbour in Lake Huron, Ontario, Canada (SV5-C) were briefly ultrasonicated (< 60 s) in Milli-Q water to disaggregate particles attached the cyst walls, sieved over a 50 µm nylon mesh sieve and retained on a 20 µm precision sieve (Storck Veco #317). Other specimens of empty dinocysts, previously isolated for germination experiments, were analyzed from the Wadden Sea (NW Germany)

and Omura Bay (Kyushu, Japan). The various geographic locations, environmental settings and isolation procedure (i.e. empty cysts isolated from the sediment matrix or empty cysts utilized shortly after hatching) were used to verify that these different situations did not overprint the cyst wall signal.

To remove extraneous apolar contaminants, all material was soaked in ethanol (EtOH) or dichloromethane (DCM) for at least 30 min. To remove extraneous water soluble molecules, all material was then rinsed three times with Milli-Q water. Similar to the procedure described in Versteegh et al. (2007) and Bogus et al. (2012), individual specimens with no visibly attached particles were identified to species level with a light microscope, isolated with a micropipette, transferred to either a salt (NaCl) plate or an Au-coated mirror, dried overnight at 60 °C, and analyzed immediately.

In total, specimens from nine extant dinocyst species from the orders Gonyaulacales, Gymnodiniales, and Peridiniales were isolated. The cyst species are produced by either phototrophic or heterotrophic dinoflagellates. Cyst names are used in the descriptions. At least three specimens of each species were picked and analyzed, and in the case of two species, specimens from two different locations were analyzed (Table 2).

Micro-Fourier transform infrared spectroscopy

Infrared spectra of specimens from GeoB 2341, GeoB 4804, and GeoB 6010 were recorded with a Nicolet FT-IR spectrometer coupled to a Nicplan microscope, a Protégé™ 460 optical bench, a mercury cadmium telluride (MCT)- A detector cooled with liquid N_2, Ever-Glo source, and a KBr beamsplitter. The adjustable apertures (upper and lower) were set at a constant area of 15 x 15 µm. Two hundred and fifty-six scans at 8 cm^{-1} resolution were obtained in transmission mode over a spectral range of 4000-650 cm^{-1}. All other specimens were analyzed with a BRUKER IFS 66v coupled to an IR Scope II equipped with a MCT detector cooled with liquid N_2 and KBr beamsplitter. Two hundred and fifty-six scans at 4

169 cm^{-1} resolution were recorded in reflection mode over a spectral range of 4000-650 cm^{-1}. The
170 replicability of FTIR analysis using these two different devices was checked through the use
171 of a chitin standard (Sigma, Lot 59F7265). The spectra proved consistent and are displayed
172 after subtracting the background of air and the bare plates (NaCl [Nicolet]; Au [Bruker]), and
173 baseline correction. Assignments of the main IR absorptions to chemical bonds (Table 3)
174 were based on Colthup et al. (1990), Coates (2000), and additional published literature.
175
176 RESULTS AND DISCUSSION
177 *Evidence for carbohydrate-based dinosporins*
178 For all species, the region between 3600-3000 cm^{-1} showed a strong and broad absorption
179 with a maximum near 3350 cm^{-1} (OH stretch) and, sometimes, a shoulder near 3270 cm^{-1}
180 and/or 3100 cm^{-1} (Table 3, Figs. 2 and 3). Absorptions were relatively weak in the 3000-2775
181 cm^{-1} region (CH stretching), except for the spectrum of Impagidinium patulum (Wall) Stover
182 and Evitt, which exhibited two stronger absorptions at 2925 cm^{-1} and 2860 cm^{-1}. Since this
183 was observed in all I. patulum specimens, it is likely an intrinsic component. The relatively
184 weak absorptions in this region suggested a minor contribution from CH_2 and CH_3 groups.
185 There was a pattern of four absorptions between 1200-1030 cm^{-1} that are highly indicative of
186 C-O stretching and the deformation vibrations of sugar rings: 1160 cm^{-1} (C-O-C asymmetric
187 vibration), 1110 cm^{-1} (glucose ring stretch), 1060 cm^{-1} (C-O stretch), and 1030 cm^{-1} (C-O
188 stretch). This series was most apparent in *Dubridinium caperatum* Reid, *Spiniferites*
189 *pachydermus* (Rossignol) Reid, *Tuberculodinium vancampoae* (Rossignol) Wall, cysts of
190 *Polykrikos schwartzii* Bütschli, and cysts of *Peridinium wisconsinense* Eddy. The remaining
191 species, *Operculodinium centrocarpum* sensu Wall and Dale, *I. patulum*, *Brigantedinium* spp.
192 Reid, and cysts of *Polykrikos kofoidii* Chatton, exhibited most of these absorptions; however,
193 the appearance of this region either differed by containing less definition, as in *O.*

194 *centrocarpum*, *I. patulum*, and *Brigantedinium* spp., or by appearing shifted, as in cysts of *P.*
195 *kofoidii*. This variability is discussed further in subsequent sections.
196 Despite the variability exhibited, the absorptions between 1200-1030 cm^{-1}, together
197 with the diagnostic absorptions at 896-902 cm^{-1}, provide a strong argument that a
198 carbohydrate with a β-glycosidic linkage (Kačuráková and Wilson 2001, Cárdenas et al.
199 2004, Versteegh et al. 2012) forms the backbone of dinosporins. The cell walls of the motile
200 stage of the freshwater dinoflagellate Peridinium westii Lemmermann were shown to contain
201 both β-1→3 and β-1→4 glycosidic bonds (Nevo and Sharon 1969). Unfortunately, the FTIR
202 spectra of β-1→3 linked glucans (e.g., Furuhashi et al. 2009) can be similar to those with β-
203 1→4 linkages (e.g., Pandey 1999) that are also apparent in the highly resolved 1000-600 cm^{-1}
204 region described by Barker et al. (1954). It is therefore not currently possible to definitively
205 determine the type of β-glycosidic linkage in the dinosporin macromolecule. Regardless, the
206 analyzed species demonstrate carbohydrate-based dinosporins and complement the
207 interpretation of a carbohydrate backbone from FTIR spectroscopy and pyrolysis gas-
208 chromatography mass spectrometry of culture- and sediment-derived Lingulodinium
209 machaerophorum (Versteegh et al. 2012) as well as FTIR analyses of the extinct genus
210 Apectodinium (Bogus et al. 2012).
211
212 *Evidence for two broadly different dinosporin groups*
213 Regardless of the evidence for a carbohydrate-based dinosporin composition in all dinocyst
214 species, it appears that at least two broadly different types of dinosporin occur. This
215 distinction was based on the pattern of absorptions between 1850-650 cm^{-1} (Figs. 2 and 3) and
216 evidence of the main functional groups present (Table 3). The first dinosporin group, here
217 referred to as Group I, includes *Impagidinium patulum*, *Operculodinium centrocarpum*,
218 *Spiniferites pachydermus*, *Tuberculodinium vancampoae* (all Gonyaulacales) and the
219 freshwater cysts of *Peridinium wisconsinense* (Peridiniales; Fig. 2). In this group,

absorptions between 1200-1030 cm^{-1} are dominant, absorptions between 1470-1200 cm^{-1} (CH_2 and CH_3 bending and rocking; OH in-plane deformation vibrations) are second in amplitude, and those between 1850-1600 cm^{-1} (adsorbed OH; conjugated C=O bonds) are the weakest. The only exception was in the spectrum of *T. vancampoae*, where the absorption at 1600 cm^{-1} is almost as strong as the 1200-1030 cm^{-1} region.

There was spectral variability within Group I, such as the changes in relative intensity between the 1640 cm^{-1} and 1600 cm^{-1} absorptions (Fig. 2). The most prominent 1640 cm^{-1} absorption is exhibited in the *S. pachydermus* spectrum, whereas it is either a shoulder or a weak peak in the other species. This pattern is reversed for the absorption at 1600 cm^{-1} where it is only a shoulder in *S. pachydermus*, but a stronger signal in *O. centrocarpum* and, especially, *T. vancampoae*. A strong peak at 1600 cm^{-1} could suggest more ester bonds in those species' cyst walls (e.g., Yuen et al. 2009). However, minor absorptions between 3000-2800 cm^{-1} and 1470-1350 cm^{-1} imply that methylene and methyl groups, and thus ester bonds, cannot be responsible for a more pronounced absorption at 1600 cm^{-1}. It is also possible that this represents the influence of aromatic skeletal vibrations, as shown in lignin (Pandey 1999), but there is no clear evidence for aromatic vibrations (~ 1500 cm^{-1}) in any of the dinocyst spectra. Therefore, at this point, a more definitive explanation of the 1600 cm^{-1} absorption is not available.

There was also variability in the deformation pattern between 1200-850 cm^{-1}. This region is comprised of four separate, defined absorptions in the cysts of *P. wisconsinense*, *S. pachydermus*, and *T. vancampoae* that closely match the spectrum of the β-linked glucan, cellulose (Fig. 2) as well as the previously published spectrum for *Lingulodinium machaerophorum* (Versteegh et al. 2012). In fact, the spectrum of *S. pachydermus* was so remarkably similar to cellulose overall that each of the absorptions observed for S. pachydermus can easily be assigned using the cellulose spectrum (Pandey 1999). The lack of this absorption series in *I. patulum* and *O. centrocarpum*, particularly the peak at 1112 cm^{-1},

246 and the existence of a broader absorption centered at 1060 cm^{-1} may indicate that glucose is
247 not the only sugar monomer present. While cellulose is the best known β-glucan (Aspinall
248 1983) and is described as the primary material comprising the theca of dinoflagellates (Sekida
249 et al. 2004), other non-cellulosic β-glucans, such as mannan, are common and well-
250 documented components in plant (e.g., Kačuráková and Wilson 2001, Burton and Fincher
251 2009) and algal cell walls (e.g., Frei and Preston 1964, Stone 2009), including the motile
252 dinoflagellate *Peridinium westii* (Nevo and Sharon 1969). Thus, it is likely that non-
253 cellulosic β-glucans also contribute to the carbohydrate signal of dinosporins in this group. In
254 general, we propose that a spectral signal indicating β-glucans represents the signature
255 composition of these dinocyst species.
256 Dinocysts in the second group (Group II) consist of *Brigantedinium* spp., *Dubridinium*
257 *caperatum* (both Peridiniales), and cysts of *Polykrikos schwartzii* and *P. kofoidii*
258 (Gymnodiniales). These species exhibited a different spectral pattern of relative absorption
259 strength, demonstrated greater heterogeneity with respect to the relative intensity of each of
260 the regions, and, most significantly, included evidence for nitrogen (N)-containing functional
261 groups (Fig. 3, Table 3). The region between 1850-1500 cm^{-1} dominated in *D. caperatum* and
262 cysts of *P. kofoidii*, but it was less intense in cysts of *P. schwartzii* and *Brigantedinium* spp.
263 Within this region, maxima occurred especially between 1585-1560 cm^{-1}, and there was a
264 clear shoulder at 1660 cm^{-1}. Absorptions between 1585-1560 cm^{-1} are characteristic of amide
265 II bonds (CN stretching and NH bending), while the shoulder at 1660 cm^{-1} in all of the
266 species probably reflects amide I bonds, which is the result of the influence of hydrogen
267 bonding (C=O···H-N; Cárdenas et al. 2004). The area between 1500-1200 cm^{-1} dominated
268 only in Brigantedinium spp., although all of the Group II dinocysts showed a small absorption
269 near 1255 cm^{-1} (NH bending). The absorptions between 1420-1370 cm^{-1} reflect CH bending,
270 and the absorption at 1312 cm^{-1} indicates CN stretching and NH bending (amide III). Further
271 evidence for the presence of nitrogen included a shoulder at 3100 cm^{-1} (NH stretching),

272 further NH stretching absorptions (~3268 cm^{-1}) likely encompassed within the broad OH
273 stretching region (3600-3000 cm^{-1}) and manifested by a shifting of the peak center (relative to
274 Group I), and a small peak at 698 cm^{-1} (NH wagging; amide V). Absorptions between 1200-
275 1030 cm^{-1} (C-O stretching) account for a much smaller proportion of the total absorptions
276 from 1850-830 cm^{-1} with the exception of cysts of P. schwartzii, where the absorptions in this
277 region dominated and clearly demonstrated the four separate absorptions characteristic of
278 sugar ring vibrations present in many of the Group I dinocysts. In combination, the spectral
279 evidence in Group II included many absorptions that are typically seen in the spectrum of the
280 polysaccharide chitin (Fig. 3a; e.g., Cárdenas et al. 2004) as well as peptides (e.g.,
281 Venyaminov and Kalnin 1990). The evidence of N-containing functional groups may reflect
282 single amino acids, as is the case for chitin, or more complex, proteinaceous material such as
283 (oligo)peptides. The combination of the carbohydrate evidence, together with the amide bond
284 evidence, suggest a dinosporin composition based on a more chitin-like glycan (Stankiewicz
285 et al. 1998, Kačuráková et al. 1999, Cárdenas et al. 2004) or even a chitin-glucan complex
286 (Šandula et al. 1999). The exhibited in-group variability could indicate different sugar
287 moieties, as postulated for Group I, suggest contributions of different amino acids
288 (Venyaminov and Kalnin 1990) or chitins (i.e. α or β, Cárdenas et al. 2004), and/or reflect
289 varying ratios of N-containing functional groups to the carbohydrate backbone.
290
291 *Explanation of compositional differences*
292 Our results demonstrate considerable spectral and, thus, compositional diversity among the
293 dinocyst species that indicate dinosporin is a chemically heterogeneous compound. The most
294 fundamental distinction between the dinosporins is the inclusion of N-containing functional
295 groups in Group II and we now explore the reasons for this difference in composition. Each
296 of the dinocyst species in Group I is produced by phototophic dinoflagellates, while the
297 Group II dinocysts are produced by heterotrophic dinoflagellates (Table 2). The heterotrophic

298 species studied here prey upon a variety of dinoflagellates and diatoms (e.g., Jacobson and
299 Anderson 1986, Matsuoka et al. 2000, Naustvoll 2000, Menden-Deuer et al. 2005).
300 Therefore, the origin of the amide groups in Group II dinosporins may be from predation by
301 the dinoflagellates, which leads to an accumulation of N-rich compounds (i.e., proteinaceous
302 compounds) within the cell as a result of prey digestion. Many heterotrophic organisms,
303 whose growth is energy limited, produce cell coverings that contain both amino acids and
304 sugars like peptidoglycans (bacteria) and chitin (arthropods and fungi) because both types of
305 compounds are abundant in prey and therefore energetically favorable to use. On the other
306 hand, phototrophic organisms are not energy limited, but nutrient limited (i.e. nitrogen and
307 phosphorus). Therefore, it is not energetically favorable for them to utilize these limited
308 nutrients to build a metabolically inactive cell covering, but rather to incorporate the products
309 of photosynthesis (e.g., Thornton et al. 1999, Wotton 2004, Ellegaard et al. 2013). As
310 dinocyst walls are assumed to be constructed using compounds from within the dinoflagellate
311 cell (Kokinos and Anderson 1995), the incorporation of photosynthetic products into a Group
312 I dinocyst would account for a glucan backbone, while the predominance of proteinaceous
313 material would lead to the inclusion of N-containing functional groups into Group II
314 dinocysts. In both cases, dinoflagellates thus use compounds that are in excess and
315 energetically favorable, determined by their nutritional strategy.
316 Compositional differences between phototrophic and heterotrophic dinoflagellates and
317 their cysts have previously been suggested by studies investigating the autofluroescence of
318 the two groups. Fluorescence microscopy was used to distinguish motile photosynthetic
319 dinoflagellates from heterotrophic ones (Lessard and Swift 1986). Additional work with
320 fossil and recent heterotrophic dinocysts demonstrated they do not exhibit autofluorescence
321 (Brenner and Biebow 2001) and this absence has been used to infer a heterotrophic ecology in
322 newly described dinocyst species (Verleye et al. 2011). Our results support these findings by

323 providing the first description of differences in chemical composition between phototrophic
324 and heterotrophic dinocysts.
325 In contrast to the expectation that dinocyst composition might solely exhibit
326 characteristic phylogenetic differences, a clear division along phylogenetic lines is absent.
327 Most of the Group I dinocysts are in the order Gonyaulacales, while Group II dinocysts are
328 either Peridiniales or Gymnodiniales; however, cysts of Peridinium wisconsinense exhibit
329 spectra firmly positioned in Group I despite being in the order Peridiniales. Both
330 compositional groups are polyphyletic when compared to dinoflagellate phylogeny (Fensome
331 et al. 1993, Taylor 2004, Zhang et al. 2007, Hoppenrath and Leander 2010, Orr et al. 2012;
332 Fig. 4). Therefore, we propose that nutritional strategy rather than phylogeny is the primary
333 factor identified which determines cyst wall composition. However, related species tend to
334 have related life strategies so some covariance with species phylogeny is expected. This is
335 most evident in Group II where the spectra of the *Polykrikos* species are more similar in terms
336 of relative absorption intensities than to the other taxa investigated in this group (Fig. 3).
337 We cannot exclude the possibility that some of the differences observed result from
338 taphonomic and environmental heterogeneity and/or preparative processes. Taphonomic
339 processes, such as sulfurization in anoxic environments (Kok et al. 2000, van Dongen et al.
340 2003, Versteegh et al. 2007) or oxidative polymerization in oxygenated settings (Versteegh et
341 al. 2004, Gupta et al. 2006, de Leeuw 2007), can overprint the original biomacromolecular
342 signal. Even though the analyzed specimens were isolated from surface sediments, they may
343 have undergone some early diagenetic alteration that led to the loss of the most easily
344 degradable components. Diagenetic effects on dinosporin composition are not well known,
345 but Versteegh et al. (2007) showed an initial preferential loss of oxygen-containing functional
346 groups as well as an increase in aliphatic CH_x bonds during diagenesis. All of our measured
347 specimens demonstrated strong absorptions for oxygen-containing functional groups and

relatively weaker absorptions for aliphatic CH_x, suggesting that diagenesis as a whole has not significantly affected the cyst wall chemistry.

Some of the variability seen between and within the two groups may also be explained by the presence, absence and/or thickness of layers with different contributions of carbohydrates and amino acids, or it may be that different proportions of these compounds remain attached to the cyst wall after hatching or migrate onto the cyst wall from the sediment. However, there was clear consistency between spectra of the same dinocyst species measured from more than one location, *Operculodinium centrocarpum* and cysts of *Polykrikos schwartzii* (Table 2, Figs. 2, 3 [gray lines]). The distinction between Group I and II dinosporins was also consistent between empty cysts of *Tuberculodinium vancampoae* and *Dubridinium caperatum* measured shortly after hatching from. The maintenance of this distinction (i.e. the absence of nitrogen-containing functional group evidence in *T. vancampoae*) indicates little effect, if any, from leftover cell material after sample treatment.

In terms of preparative alteration, the specimens analyzed all represent visibly and chemically clean, but thermally untreated material. Therefore, the origin of the amide bonds cannot be artifacts, such as melanoidin-like polymers, produced during preparation (Allard et al. 1997, 1998). Cleaning involved gentle ultrasonic treatment (< 60 s; Mertens et al. 2009a) and solvent extraction, specifically washing with Milli-Q water to remove water soluble contaminants, and DCM/EtOH to remove apolar contaminants. The use of solvent extraction alone, as opposed to its use in conjunction with acid and/or base hydrolysis (e.g., Kokinos et al. 1998, Versteegh et al. 2012), was used for a couple of reasons. First, even a short treatment with a base selectively destroys peridinialean cysts (e.g., Dale, 1976, Mertens et al. 2009a). Additionally, a comparison between extracted and hydrolyzed cultured *Lingulodinium machaerophorum* cysts and extracted sediment-derived cysts showed remarkably similar spectra (Versteegh et al. 2012), suggesting that hydrolysis was not necessary to render the cysts chemically clean. The *L. machaerophorum* spectra are very consistent with the Group I

spectra (Fig. 2). It may not be possible to fully discount effects of invisible material on/in cyst walls insoluble in water and organic solvents on resulting spectra, particularly for species with significant surface ornamentation such as *O. centrocarpum*, *T. vancampoae*, *Spiniferites pachydermus* and the *Polykrikos* species, but the lack of evidence for an overprint indicates this is unlikely; instead, it suggests that preparing cysts with solvent extraction has the potential for wide applicability as even delicate cysts with lower resistance to harsh chemical treatments will be able to be reliably analyzed.

The aforementioned observations indicating (1) little to no diagenetic effects, (2) consistency between the same species analyzed from multiple locations as well as the maintenance of the group-wise distinction between different species of freshly hatched cysts, and (3) the similarity of the Group 1 spectra to spectra from hydrolyzed *L. machaerophorum* cysts argue against any significant overprint from diagenesis, environment and sample preparation.

Implications for (paleo)environmental studies

A determination of two broadly different dinosporin types was based on the FTIR spectral patterns and functional groups present, and attributed to nutritional strategy. The spectral variability, particularly with regards to the relative strength of the individual absorptions, suggests that the story is likely more complicated given the large variety in nutritional strategies amongst dinoflagellates (Schnepf and Elbrächter 1993) as well as previous evidence that aspects of dinosporin composition are also likely taxon-specific (de Leeuw et al. 2006, Bogus et al. 2012, Figs. 2, 3). So, the spectral variability within the two groups does not exclude a phylogenetic component in dinosporin synthesis, only that it is obscured in this case by the complex interplay between the dinoflagellate and its environment. Thus, it may be more accurate to define dinosporin overall as a suite of chemically distinct but related carbohydrate-based biomacromolecules. The most apparent distinction in a broad sense is the

inclusion of amide groups into some species' dinosporins, resulting from heterotrophy, and in this sense dinosporin does not follow a strict delineation with phylogeny (Fig. 4). Therefore, based on the evidence, we suggest FTIR spectra of dinocysts have the potential to assess (paleo)nutritional strategies. This may prove especially interesting for species that may change their nutritional strategy in response to environmental conditions.

Very important subsequent steps are to expand the number of investigated taxa in general and through the sedimentary record to robustly determine the predictive value of dinosporin composition for (paleo)ecology. Even though carbohydrates are the most abundant form of biomass on earth (Kurita 2006), they are usually considered labile compounds (e.g., de Leeuw and Largeau 1993, Arnosti 1995) with preservation determined by the lability of individual sugar monomers (Moers et al. 1994, Marchand et al. 2009) and diagenetic alteration, such as macromolecular skeletal rearrangements (Almendros et al. 1997). There is evidence for carbohydrate preservation relatively far back in the sedimentary record ([Miocene] e.g., Lechien et al. 2006), including well-preserved glycolipids ([Eocene] Bauersachs et al. 2010) as well as spectra demonstrating good preservation of Group I compositional signals ([late Paleocene] Bogus et al. 2012). Dinosporin is likely to involve some inherent significant structural differences to straightforward carbohydrates, such as having a more highly cross-linked backbone (Versteegh et al. 2012), which explains the generally high preservation potential of dinocysts. These structural differences are unfortunately not resolvable in FTIR spectra. However, the *L. machaerophorum* cysts were analyzed for structural information, which indicated a carbohydrate-based polymer (Versteegh et al. 2012). Due to the strong similarities between *L. machaerophorum* and Group I spectra (Fig. 2), it is probable that the cyst macromolecule is comparable.

Selective preservation of dinocysts in the sedimentary record has been suggested (Zonneveld et al. 1997, 2001, Combourieu-Nebout et al. 1998, McCarthy et al. 2000, Versteegh et al. 2010), although it is likely not a straightforward process (Reichart and

426 Brinkhuis 2003). Based on these studies peridinialean cysts have been considered more
427 sensitive to oxidation than gonyaulacalean cysts, which is supported by laboratory evidence
428 that peridinialean cysts are destroyed after harsh base treatment (e.g., Dale 1976, Hopkins and
429 McCarthy 2002, Mertens et al. 2009a). However, based on dinosporin composition, we
430 suggest that sensitivity to oxidation is more likely predicated on dinosporin composition. For
431 example, the peridinialean cysts of *P. wisconsinense* withstand treatment by harsh bases
432 during palynological preparation unlike many marine peridinialean species; there are also
433 indications they are more prevalent in sedimentary successions than previously recognized
434 (e.g., Miller et al. 1982, Zippi et al. 1990, McCarthy et al. 2011, Mertens et al. 2012).
435 Therefore, the Group I dinocysts may be inherently more resistant while the Group II
436 dinocysts are more heavily reliant on additional diagenetic factors, such as sulfurization or
437 skeletal rearrangement, to facilitate preservation. It is also interesting to note that the Group
438 II cysts are brown in color, while Group I dinocysts are transparent, suggesting the possibility
439 that the inclusion of nitrogen-containing functional groups and/or different pigments may also
440 contribute to increased oxidative sensitivity. Regardless, these compositional differences
441 between dinosporins provide the first empirical evidence that differences in preservation
442 potential may be related to dinocyst wall chemistry.
443
444 CONCLUSIONS
445 Micro-FTIR spectroscopic analyses of recent dinoflagellate resting cyst (dinocyst) walls from
446 three large orders (Gonyaulacales, Gymnodiniales, Peridiniales) showed that all of the
447 dinocysts had a carbohydrate-based macromolecular composition. Spectral and
448 compositional variability indicated that the cyst wall macromolecule (dinosporin) is a
449 collection of chemically distinguishable but related biomacromolecules determined by the
450 interaction of the cyst-producing dinoflagellate with its environment. Two groups were
451 defined on the basis of this spectral variability. The first group encompasses dinocysts

produced by phototrophic dinoflagellates, which includes all the gonyaulacalean cysts and the peridinialean cysts of *Peridinium wisconsinense*. Their dinosporin has a β-glucan backbone, which concurs with a previous study of *Lingulodinium machaerophorum* (Versteegh et al. 2012). The second group consists of dinocysts produced by heterotrophic dinoflagellates, including the remainder of the peridinialean and all of the gymnodinialean cysts studied. They showed the presence of amide bonds within their dinosporins. These systematic differences reflect the nutritional strategy rather than the phylogeny of the cyst-producing dinoflagellate and may result from the incorporation of chemical compounds in excess within the cell into dinosporins. Thus, the nitrogen-containing functional groups present in the heterotrophic dinosporins may originate from the digestion of prey and the resulting proteinaceous, nitrogen-rich material. The nutritional strategies of the cyst-producing dinoflagellates were polyphyletic, as demonstrated by the grouping of the peridinialean cysts of *P. wisconsinense* with the other phototrophic species (all Gonyaulacales) as well as the grouping of remaining Peridiniales and Gymnodiniales cysts. As nutritional strategy, not phylogeny, appears to be the primary factor determining dinosporin composition, cyst wall chemistry may show potential as a paleoecological proxy by inferring the past nutritional strategies of extinct taxa. Finally, the compositional differences between the two groups suggest that preservation potential is influenced by dinosporin composition.

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881 *Tables*

882 Table 1: Surface sediment and culture sample information from which the dinocyst specimens

883 were isolated.

#	Name	Location	Latitude	Longitude	Water depth (m)	Setting	Reference
1	GeoB 2341	Benguela upwelling	31°55’48”S	18°12’36”E	84	Marine	Holzwarth et al. (2007)
2	GeoB 4804	Benguela upwelling	24°8’60”S	12°40’12”E	2090	Marine	Holzwarth et al. (2007)
3	GeoB 6010	NE Atlantic (off NW Africa)	30°15’N	10°2’16.8”W	406	Marine	Holzwarth et al. (2010)
4	SV5-C	Honey Harbour (Lake Huron, Canada)	44°52'26"N	79°48'55"W	19.4	Lacustrine	McCarthy et al. (2011)
5	Schillig	Wadden Sea (NW Germany)	53°42’56”N	7°58’11.93”E	0.5	Culture	-
6	GeoB 1010	Omura Bay (Kyushu, Japan)	33°N	128°49’48”E	Not stated	Culture	Zonneveld and Susek (2007)

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886 Table 2: Dinocyst species analyzed in this study, their motile affinities, and nutritional

887 strategy with reference to the respective studies.

Sample*	Specimens measured (#)	Dinocyst species	Motile affinity	Order
2	3	*Impagidinium patulum* (Wall) Stover and Evitt	*Gonyaulax* sp. Diesing[a]	Gonyaulacales
2,3	6 (3 from each location)	*Operculodinium centrocarpum* sensu Wall and Dale	*Protoceratium reticulatum* (Claparède *et* Lachmann) Bütschli[b]	Gonyaulacales
2	4	*Spiniferites pachydermus* (Rossignol) Reid	*Gonyaulax* sp. Diesing[c]	Gonyaulacales
6	3	*Tuberculodinium vancampoae* (Rossignol) Wall	*Pyrophacus steinii* (Schiller) Wall and Dale[d]	Gonyaulacales
4	3	Cyst of *Peridinium wisconsinense* Eddy	*Peridinium wisconsinense* Eddy[e]	Peridiniales
1	4	*Brigantedinium* spp. Reid	*Protoperidinium* sp. Bergh[c]	Peridiniales
5	3	*Dubridinium caperatum* Reid	*Preperidinium meunieri* (Pavillard) Elbrächter[f]	Peridiniales
1	4	Cyst of *Polykrikos kofoidii* Chatton	*Polykrikos kofoidii* Chatton[g]	Gymnodiniale
1,2	6 (3 from each location)	Cyst of *Polykrikos schwartzii* Bütschli	*Polykrikos schwartzii* Bütschli[g]	Gymnodiniale

888 *Sample numbers correspond to Table 1. [a]Rochon et al. (1999). [b]Matsuoka et al. (1997).

889 [c]This study. [d]Matsuoka (1985). [e]McCarthy et al. (2011). [f]Matsuoka (1988); see Elbrächter

890 (1993) for synonymy. [g]Matsuoka et al. (2009). [h]Gómez (2012).

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893 Table 3: Assignments of major FTIR absorptions present in Group I (phototrophic) and

894 Group II (heterotrophic) dinosporins. See Table 2 for each species' nutritional strategy.

Dinocysts	Wavenumber (cm^{-1})	Assignment	Comments
Group I			
	3348	νOH	
	2925	νCH	
	2860	νCH	
	1640	νC=O	
	1600	νC=C + νC=O	
	1430	δCH_2	
	1370	$\delta CH + \delta C\text{-}CH_3$	
	1318	δOH	
	1163	νC-O-C	
	1112	νC-O	Glucose ring
	1059	νC-O	
	1033	νC-O	
	897	γCH	β-glycosidic bond
Group II			
	3479, 3448, 3426	νOH	
	3268	νNH	
	3106	νNH	
	2965-2860	νCH	
	1660, 1627	νC=O	Amide I
	1585-1550	νCN+ δNH	Amide II
	1420	δCH_2	

	1370	δCH + δC-CH_3	
	1312	νCN+ δNH	Amide III
	1255	δNH	
	1157	νC-O-C	Ring
	1113	νC-O	Glucose ring
	1060	νC-O	
	1027	νC-O	
	896, 902	γCH	β-glycosidic bond
	875-746	ρCH2	
	698	γNH	Amide V

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Figure captions

Figure 1: Locations of the marine and lacustrine surface samples analyzed in this study.

Figure 2: FTIR spectra of the Group I (phototrophic) dinocysts compared to *Lingulodinium machaerophorum* from sediment (black line) and culture (gray line; Versteegh et al. 2012) and cellulose (Pandey 1999). The gray *O. centrocarpum* spectrum denotes the analysis from the second measured location (see Tables 1, 2). Absorption assignments are given in Table 3.

Figure 3: FTIR spectra of the Group II (heterotrophic) dinocysts and the chitin standard. The gray line for cysts of *P. schwartzii* reflects spectral data from a second location (see Tables 1, 2). Absorption assignments are given in Table 3.

Figure 4: Phylogenetic subdivision of the major orders according to phylogenies proposed by Fensome et al. (1993) and Taylor (2004) and comparison with the two groups derived from micro-FTIR measurements.