1 **Effects of legacy metabolites from previous ecosystems on the environmental metabolomics**

2 **of the brine of Lake Vida, East Antarctica**

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Abstract

Lake Vida, located in an closed basin in the McMurdo Dry Valleys, East Antarctica, permanently encapsulates an interstitial anoxic, aphotic, cold (-13°C), brine ecosystem within 27+ m of ice. Metabolically active, but cold-limited, slow-growing bacteria were detected in the brine. Lake Vida brine is derived from the evaporation of a body of water that occupied the same basin prior to ~2800 years ago. The characteristics of this body of water changed over time and, at one point, likely resembled other modern well-studied perennial ice-covered lakes of the dry valleys. We characterized the dichloromethane-extractable fraction of the environmental metabolome of Lake Vida brine in order to constrain current and ancient biogeochemical processes. Analysis of the dichloromethane-extract of Lake Vida brine by gas chromatography-mass spectrometry and comprehensive multidimensional gas chromatography-time of flight-mass spectrometry reveals the presence of legacy compounds (i.e. diagenetic products of chlorophylls and carotenoids) deriving from photosynthetic algae and anaerobic, anoxygenic photosynthetic bacteria. This legacy component dilutes the environmental signal of metabolites deriving from the extant bacterial community. The persistence of legacy metabolites (paleometabolites), apparent in Lake Vida brine, is a result of the slow turnover rates of the extant bacterial population due to low metabolic activities caused by the cold limitation. Such paleometabolites may also be preserved in other cold-limited or nutrient-depleted slow-growing ecosystems. When analyzing ecosystems with low metabolic rates, the presence of legacy metabolites must first be addressed in order to confidently recognize and interpret the environmental metabolome of the extant ecosystem.

1. Introduction

Environmental metabolomics encompasses a subset of the field of metabolomics that is used to elucidate the relationship between living organisms and their environment by characterizing the global pool of metabolites obtained directly from that environment (Viant, 2007; Bundy et al., 2009). The biological interaction between a community and its environment is expressed in a suite of metabolites that contributes to the total organic carbon pool of the ecosystem, and is a reflection of both the current environmental conditions and the genetic potential of that community (Kido Soule et al., 2015). In lacustrine ecosystems, microbial activity is largely responsible for the degradation and reworking of dissolved organic material (Meyers and Ishiwatari, 1993). Applying environmental metabolomics to lacustrine ecosystems can provide insight into the current metabolic activities of its microbial community. Combined with other meta-"omics" platforms, such as metagenomics or metatranscriptomics, environmental metabolomics can illuminate the effects of ecosystem stressors such as temperature, salinity, or nutrient limitation on metabolic pathways (Bundy et al., 2009), allowing for an unprecedented view of the biogeochemistry of the ecosystem of interest, thus providing constraints on the processes active in a community under specific environmental conditions.

We hypothesize that the standing crop of metabolites in a cold-limited, slow-growing ecosystem may contain both the metabolites derived from the active community as well as metabolites derived from past environmental conditions (a legacy component). The co-occurrence of metabolites corresponding to current biological processes with those corresponding to legacy potentially complicates the interpretation of the environmental metabolomics data as the metabolites must first be recognized as part of a legacy component, a modern component, or as part of both. However, if metabolites corresponding to legacy can be

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distinguished from those deriving from current biological activity, legacy metabolites

(paleometabolites) may provide useful information on past biological processes that otherwise

may not be preserved in the limnological record. The contribution of legacy metabolites is likely

to be observable in the cryosphere where fluxes of new metabolites may be minute relative to the

amount of metabolites inherited from previous ecosystems.

All microbial communities in the McMurdo Dry Valleys (MDVs), East Antarctica (Fig

1A), are challenged by environmental stress imposed by cold temperatures, and seasonal

variation of sunlight (Priscu et al., 1999). Aquatic ecosystems in the MDVs are typically

dominated by microorganisms and are influenced by bottom up controls such as availability of

resources and environmental conditions, rather than by top down processes such as predation and

competition (Moorhead et al., 1999). For example, in perennially ice-covered lakes, the spatial

and temporal variation in light, oxygen, salinity and nutrients significantly affects the community

structure. In addition, in the bottom waters of these lakes, the biological activity is strongly

constrained by the chemical inventory, notably dissolved inorganic carbon (DIC) and dissolved

organic carbon (DOC), that persists in the environment but is derived from processes associated

with previous ecosystem conditions (Moorhead et al., 1999; Lyons et al., 2000; Knoepfle et al.,

2009).

Lake Vida, located in Victoria Valley, the northernmost valley in the MDVs (Fig. 1B),

permanently encapsulates an aphotic, anoxic, very cold (-13.4°C), interstitial brine (salinity 188)

within its >27 m of ice (Fig 1C; Murray et al., 2012; Dugan et al., 2015b). Subsurface imaging of

Lake Vida revealed that the brine network may extend up to 100 m deep (Dugan et al., 2015a).

Radiocarbon ages of microbial organic matter obtained at 12 m beneath the surface of the ice

cover suggest that Lake Vida brine (LVBr) has been isolated from the environment for at least

90   ~2800 ^{14}C y (Doran et al., 2003). The total concentration of DOC in LVBr is high (580 mg-C·L^{-}

91   ^{1}; Cawley et al., 2016). LVBr dissolved organic matter (DOM) has older radiocarbon ages (2955

92   to 4150 ^{14}C years BP; Cawley et al., 2016) than the microbial mat sampled at a depth of 12 m in

93   the ice cover (2770±120 ^{14}C years BP; Doran et al., 2003). Spectroscopic and elemental

94   characterization of LVBr DOM revealed low aromaticity, with predominantly hydrophilic

95   microbial exudates (proteins, carbohydrates, amino acids, or fatty acids) that are high in N and S,

96   suggesting that most of the organic material of LVBr is derived from ancient microbial

97   production and have been further altered in the brine (Cawley et al., 2016). Isotopic analyses of

98   iron and sulfur species in LVBr suggest the presence of a tightly coupled nitrogen-iron-sulfur

99   cycle driven by both biotic and abiotic redox reactions (Proemse et al., 2017).

100   The history of the lake in the Vida basin in the last 5000 years BP is not well constrained

101   but may have included the drawdown of a lake larger than the current Lake Vida into a brine and

102   the cryoencapsulation of that brine in ice ~2800 years BP (Doran et al., 2003; Dugan et al.,

103   2015b). The existence of a body of water occupying the Vida basin that was exposed to sunlight

104   and the atmosphere is likely, given our current understanding of the age and composition of the

105   brine organic material (Cawley et al., 2016) as well as Lake Vida's recent hydrological history

106   (Dugan et al., 2015b). It is important to note that ice of Lake Vida accreted from the top of the

107   ice cover, as it is regularly (but not annually) covered by additional ice formed from meltwater

108   derived from the nearby alpine glaciers and ice sheet terminus (Dugan et al., 2015b; Doran et al.,

109   2003). Under past light-penetrating conditions, the organisms responsible for autotrophic carbon

110   fixation that would have inhabited the ancient Lake Vida were likely dominated by phototrophic

111   communities, much like most other modern perennially ice-covered lakes in the MDVs (Priscu et

112   al., 1999).

113   The present day LVBr hosts a bacterial community (neither eukaryotes nor archaea were

114   detected) that is temperature limited, such that the average generation time was estimated at 120

115   years (Murray et al., 2012). This rate is comparable to the value predicted for maintaining

116   metabolism at LVBr temperature (Price and Sowers, 2004). Due to the brine's unusual and

117   complex geochemistry (i.e. high levels of reduced and oxidized nitrogen species present as NH_4^+,

118   NO_3^-, NO_2^-, and N_2O, dissolved metals like Fe, and elevated amounts of sulfur dominated by

119   SO_4^{2-} and a very high load of dissolved organic carbon), it is difficult to precisely determine

120   which of the geochemical resources allow for the persistence of this very slow-growing

121   microbial community.

122   In subzero ecosystems such as LVBr, the carbon inventory is being processed extremely

123   slowly. As a result, the total pool of organic compounds in the brine should reflect a combination

124   of past and present day biological processes. The past, or legacy, contribution would be sourced

125   from organisms that lived in the lake's previous ecosystem, as well as the capacities of past

126   conditions to result in biotic or abiotic alteration of the organic material. In addition, organic

127   matter from the brine should also contain a molecular signal from the active microbial

128   assemblage, expressed as "modern metabolites". Since the amount of accumulated legacy

129   material in a given system is dependent on the metabolic rate and capacity for the extant

130   community to further degrade existing organic material, we speculate that other cold-limited or

131   nutrient-limited ecosystems with slow metabolic rates, such as cryopegs (permafrost brine

132   lenses), or deep subsurface environments, may also contain legacy compounds.

133   Permafrost constitutes a major portion of the cryosphere and comprises habitats that host

134   microbial communities at subzero temperatures (see review by Jansson and Taş, 2014). Cryopegs

135   are pockets of brine lenses in permafrost that are characterized by a high dissolved-solids

136 content, which prevents the liquid reservoir from freezing (temperature of -6 to -11°C and

137 salinity of 115 to 300 ppt; Gilichinsky et al., 2005; Colangelo-Lillis et al., 2016). Bacteria

138 isolated from a Siberian cryopeg have been shown to be metabolically active in the laboratory at

139 -10°C at very low rates (at $\sim 7 \times 10^{-4}$ per hour; Bakermans et al., 2003). *In situ* activity or direct

140 observation of cell reproduction in cryopegs have yet to be demonstrated. Nevertheless, bacterial

141 communities in isolated cryopegs likely remained viable for thousands of years by limiting their

142 biological activities to sufficient levels for cellular repair such as DNA damage from background

143 radiation or amino acids racemization (Bakermans et al., 2003; Gilichinsky et al., 2003).

144   The "deep subsurface" harbors vast reservoirs of microbial life (see review by Edwards et

145 al., 2012). The continental subsurface is oligotrophic and energy limited, a significant difference

146 from LVBr. However, various forms of energy, such as organic matter for

147 chemoorganoheterotrophy, are bioavailable in sedimentary rocks or brought from the surface by

148 recharging groundwater. In addition, in the continental subsurface, carbon source such as CO_2 or

149 CH_4, and redox species such as H_2, Fe^{3+}, or SO_4^{2-} have been shown to support

150 chemolithoautotrophy (Chapelle and Lovley, 1990; Amend and Teske, 2005). On the other hand,

151 the deep marine subsurface habitats consist of subseafloor sediments and crustal basement rocks

152 with energy deriving from organic matter accumulated during marine burial, methane hydrates,

153 or hydrogen produced by water-rock reactions (Chapelle et al., 2002; D'Hondt et al., 2002).

154 Nevertheless, the metabolic rates of microbial life in both continental and marine settings are

155 fundamentally limited by the fluxes of these forms of energy over time, which may be very slow

156 due to diffusion-limited processes (Pedersen, 2000).

157   Price & Sowers (2004) compared metabolic rates of microbial communities from various

158 cold surface, deep subsurface, and aeolian environments in order to calculate the dependence of

159   metabolic rate on temperature. They found that the organisms living in the deep subsurface such

160   as deep aquifers (up to 388 m; Chapelle & Lovley, 1990), deep subsurface marine sediments (up

161   to ~400 m; D'Hondt et al., 2002) and deep ice cores (~3000 m; Tison et al., 1998) are likely only

162   using their energy towards survival (i.e. repair of DNA and protein damage) rather than for

163   growth or maintenance (i.e. osmotic regulation, cellular pH maintenance, or motility). The

164   metabolic rates of deep subsurface communities are ~1 to 2 orders of magnitude lower than that

165   measured for LVBr microbes (Price and Sowers, 2004).

166   In this paper, we describe a liquid-liquid dichloromethane (DCM) extract of LVBr that

167   was analyzed by gas chromatography-mass spectrometry (GC-MS) and comprehensive two-

168   dimensional gas chromatography-time of flight-mass spectrometry (GC×GC-TOF MS). We

169   discuss the presence of legacy compounds in this fraction of the LVBr metabolite collection. We

170   assess the origin of the compounds detected on the basis of their structure and inferred biological

171   origin, and attempt to elucidate some of the past metabolic processes they represent. Finally, we

172   discuss the importance of discerning legacy metabolites from those produced by current

173   biological processes, not only for the environmental metabolome of LVBr microbial assemblage,

174   but also other environments where energy limitation, cold temperatures, or nutrient limitation

175   results in slow-growing ecosystems.

176

177   **2. Sample Collection and Methods**

178   *2.1 Brine collection*

179   During the 2010 expedition to Lake Vida, brine samples were obtained at a depth of 16 m

180   in a 18.5 m borehole in the >27 m lake ice (Fig. 1C; Murray et al., 2012; Dugan et al., 2015b).

181   The clean access sampling strategy of the brine is described in Doran et al. (2008). LVBr was

182 collected using a stainless steel submersible pump using polytetrafluoroethylene (PTFE) tubing,

183 and stored in sterile PTFE bottles spiked with $HgCl_2$ (4 mM) to prevent further biological

184 activities during storage at 4˚C.

185

186 *2.2 Solvent-based environmental metabolome extraction*

187   The total DCM-extractable fraction of LVBr (200 mL) was obtained by liquid-liquid

188 extraction. Milli-Q® water was first extracted three times with DCM. This clean Milli-Q® water

189 (100 mL) was then added to the brine to enhance the density difference between the salty

190 aqueous phase and the denser DCM phase. The DCM-soluble fraction of the brine was then

191 extracted three times using 100 mL of DCM per extraction. The extract was then rotary-

192 evaporated to near dryness and subsequently dried under a low flow of N_2. Samples were then

193 dissolved with cyclohexane (1 mg per mL of extract) and injected (1 μl) into the GC .

194

195 *2.3 GC-MS and GC×GC-TOF MS*

196   For GC-MS, a Hewlett Packard 6890 GC coupled to a HP-5973 mass selective detector,

197 with an electron ionization mode at 70 eV and helium (>99.999% ultra-high purity, Praxair®) as

198 a carrier gas with constant flow at 1 mL·min^{-1}, was used for the initial characterization of the

199 DCM-soluble LVBr extract. The chromatographic column used in the GC was a 30 m long

200 Agilent HP-5 MS (polydimethylsiloxane-95%/phenyl-5%; 0.25 mm I.D., 0.25 μm film

201 thickness). The range of the masses scanned was *m/z* 40 to *m/z* 650 at a rate of three scans per

202 second. The GC injector was operated in pulsed splitless mode at 320°C (40 psi). The oven

203 temperature was kept at 60°C for 2 minutes, then ramped at 10°C·min^{-1} to 150°C and further

204 ramped at 3°C·min^{-1} to 320°C and kept at 320°C for 20 minutes.

205

206   Due to the complexity of the DCM-soluble LVBr extract, we employed the use of a

207   GC×GC-TOF MS, which provides a higher chromatographic resolving power and detection

208   sensitivity that is suitable for separating low molecular weight analytes. The GC×GC-TOF MS

209   used is a Leco Pegasus 4D system, which consists of an Agilent 7890 GC with a split/splitless

210   injector, two capillary columns, a liquid nitrogen-cooled pulsed jet modulator, and a time-of-

211   flight mass spectrometer. The DCM-soluble LVBr extract described above was dissolved in

212   cyclohexane at 1 mg/mL and injected (1uL) into the GC×GC in pulsed splitless mode at 250°C

213   (60 sec purge time). The first capillary column was a nonpolar 5% phenyl polydimethylsiloxane

214   (Agilent BPX-5, 24.47 m; 0.25 mm ID; 0.25 μm film thickness). The oven temperature for the

215   first column was kept at 40°C for 1 minute, then ramped at 3°C·min^{-1} to 300°C, and held for 10

216   mins. The secondary capillary column was a medium-polarity 50% phenyl/50%

217   polydimethylsiloxane (Agilent BPX-50; 1.65 m; 0.1 mm ID; 0.1 μm film thickness). The oven

218   temperature of the second column was programmed to remain 10°C hotter than that of the first

219   oven. A 0.21 m, 0.1 mm ID BPX-50 was used as a transfer line to the TOF detector. Helium

220   (>99.999% ultra-high purity, Praxair®) was used as the carrier gas in constant flow of

221   1 mL·min^{-1}. The GC×GC modulation period was 6 s with a hot pulse time of 0.5 s and cool time

222   of 2.5 s. The system was coupled to a mass spectrometer with an electron ionization mode at 70

223   eV and an ion source temperature of 200°C. The solvent delay was 660 seconds and the spectra

224   were collected from *m/z* 30 to *m/z* 500 at a rate of 200 spectras per second. ChromaTOF, a Leco

225   software package, was used for data processing, which included deconvolution algorithm and

226   baseline correction. Mass spectra of reported compounds were characterized using the National

227 Institute of Standards and Technology (NIST) reference library and comparison to spectra

228 published in the literature.

229

230 **3. Results & Discussion**

231 Upon GC-MS, the total ion current (TIC) of the DCM-extractable environmental

232 metabolome of LVBr displayed an unresolved complex mixture (UCM) in which several

233 compounds can be resolved (Fig. 2). This UCM consisted mainly of coeluting low molecular

234 weight compounds (C_5 to C_{16}). The compounds that could be resolved were tentatively identified

235 based on their mass spectral fragmentation pattern, their relative retention time, and comparison

236 of their spectra with those published in the literature. In addition, utilization of GC×GC-TOF

237 MS allowed for the separation of overlapping peaks in the UCM and, therefore, the tentative

238 identification of additional compounds with structural similarities that belong to the same

239 families (Fig. 3). Below, we describe the major molecular families of compounds that comprised

240 the DCM-extractable LVBr environmental metabolome.

241

242 *3.1 Chlorophylls derivatives:*

243 A family of maleimides (1*H*-pyrrole-2,5,diones) was tentatively identified upon GC-MS

244 on the basis of mass spectral fragmentation and retention time: 3-methyl-4-ethyl-maleimide

245 (Me,Et), 3,4-dimethyl-maleimide (Me,Me) and 3-methyl-4-propyl-maleimide (Me,Pr; Fig. 2B,

246 Martin et al., 1980; Grice et al., 1996, 1997). Fig. 2B shows the summed mass chromatogram

247 *m/z* 111, 125, 139, and 153 in GC-MS, representing the major molecular ions for members of the

248 maleimide family. The same summed mass chromatogram with addition of *m/z* 67, the major

249 fragmentation ion for most of the maleimides, as well as *m/z* 97, the molecular ion for maleimide

250 (H,H) obtained upon GC×GC-TOF MS is shown in Fig. 4. The UCM formed upon GC-MS

251 masked the presence of some maleimides that were resolved upon GC×GC-TOF MS, allowing

252 for their tentative identification (Fig. S1). In particular, maleimide (H,H), present in low

253 abundance (Fig. 4A), as well as an additional family of 2,5-pyrrolidinedione (succinimides), the

254 saturated counterparts of maleimide (Fig. 5), were tentively identified (Fig. S2). Interestingly, the

255 succinimide corresponding to Me,Pr maleimide, was not observed.

256   Maleimides are degradation products of tetrapyrrole pigments commonly attributed to

257 chlorophylls and bacteriochlorophylls (Grice et al., 1996, 1997; Pancost et al., 2002; Naeher et

258 al., 2013). The distribution of maleimides in environmental samples is dependent on the original

259 abundance and diversity of their parent chlorophyll molecules (Grice et al., 1996). The formation

260 of Me,Et and Me,Me maleimides is typically a result of the oxidation of the tetrapyrrole ring of

261 chlorophyll *a* (Fig. S5, Structure **I**), which has either methyl or ethyl substituents at C_2 (Grice et

262 al. 1996, 1997). In contrast, based on structural grounds, Me,n-Pr and Me,*i*-Bu maleimides are

263 considered to be derived from bacteriochlorophyll *c*, *d*, or *e* (Fig S5, **II**), which contain various

264 alkyl substituents at C_8 (Me, Et, n-Pr, *i*-Bu, and neo-Pent), C_{12} (Me, Et), and C_{20} (Grice et al.,

265 1996, 1997; Pancost et al., 2002; Naeher et al., 2013). Me,n-Pr maleimide, however, may have a

266 chlorophyll *a* origin if the C_{17} ester undergoes hydrolysis during diagenesis (Verne-Mismer et

267 al., 1986). Bacteriochlorophyll *c*, *d*, and *e* are uniquely synthesized by the green sulfur bacteria

268 Chlorobiaceae (Gloe et al., 1975).

269   GC×GC-TOF MS allowed for the observation of a family of succinimides (Fig. 5),

270 which were originally unresolvable using GC-MS. The 2,5-pyrrolidinedione (H,H), 3,4-

271 dimethyl-2,5-pyrrolidinedione (Me,Me), and 3-methyl-4-ethyl-2,5-pyrrolidinedione (Me,Et) bear

272 structural similarity to maleimides and are suggested here to be the saturated reduction products

273   of maleimides. Whether the loss of unsaturation is caused by abiotic diagenetic processes or is an

274   enzymatically driven process remains unclear. To the best of our knowledge, this study is the

275   first to detect succinimides that are associated with maleimides in an environmental sample.

276   It is likely that the Me,Et and Me,Me maleimides in LVBr are derived from chlorophyll *a*

277   produced by photosynthetic organisms that occupied the Lake Vida basin prior to the

278   evaporation and cryoencapsulation of Lake Vida brine. On the other hand, Me,Pr maleimide in

279   LVBr most likely originated from bacteriochlorophylls *c*, *d*, or *e*, though an origin from

280   chlorophyll *a* cannot be completely ruled out. H,H maleimide was previously detected via

281   GC×GC-TOF MS in the sediment of a monomictic lake with an anoxic hypolimnion, in

282   association with other maleimides observed in this study, as well as Me,*i*-Bu (Naeher et al.,

283   2016). As Me-Pr maleimide in LVBr is derived from Chlorobiaceae (Grice et al., 1996, 1997), it

284   would suggest that the former environmental conditions at Lake Vida had, at some point, a

285   stratified water column with sulfidic bottom waters reaching into the photic zone. Chlorobiaceae

286   have been observed in Ace Lake, a permanently stratified Antarctic lake with anoxic bottom

287   waters (Hopmans et al., 2005; Ng et al., 2010), as well as several other meromictic lakes and

288   permanently stratified fjords in the Vestfold Hills, Antarctica (Burke and Burton, 1988).

289   Alternatively, some of these bacteriochlorophylls may derive as well from benthic mats such as

290   those observed by Jungblut et al. (2016) in Lake Fryxell, another perennially ice-covered lake of

291   the MDVs. Because maleimides are degradation products of photosynthetic pigments, we

292   suggest that the parent compounds were not synthesized by the current Lake Vida microbial

293   assemblage since the brine is aphotic (Murray et al., 2012).

294   Two lines of evidence point to an aphotic brine: (1) the ice-cover that encapsulates LVBr

295   contains several layers of thick sediments (>10 cm; Fig 1C), preventing any sunlight from

296   reaching the brine (Dugan et al., 2015b), and (2) the analysis of small ribosomal subunit rRNA

297   of LVBr reveals the presence of bacterial taxa that are known to grow heterotrophically,

298   chemolithoautotrophically, or using fermentation, but not photosynthetically (Murray et al.,

299   2012). Thus, the maleimides detected in the brine must have originated from the chlorophylls

300   produced by the photosynthetic community in the water column or the benthos of the lake during

301   past environmental conditions.

302   Maleimides can form directly from the photooxidation of chlorophylls in the absence of

303   enzymatic activity (Rontani et al., 1991), though it has been argued by Hendry et al. (1987) that

304   the transformation from pheopigments, macrocyclic rings that result from chlorophylls

305   breakdown via the loss of magnesium, phytol, or modification of the sidechain to smaller N-

306   bearing fragments could be enzymatically driven if light and oxygen are present. Regardless of

307   the mechanism, the transformation from chlorophylls to maleimides seem to only happen in

308   photic and oxic environments (Hendry et al., 1987; Rontani et al., 1991). Therefore, maleimides

309   in LVBr must have formed before the brine got encapsulated and became anoxic and aphotic.

310   Thus, maleimides should be considered paleometabolites, a legacy signature that is not part of

311   the modern environmental metabolome of the LVBr microbial assemblage.

312

313

314   *3.2 Carotenoid derivatives*

315   A number of compounds observed in the DCM-extractable metabolome of LVBr were

316   derived from the breakdown products of various carotenoids. Three known carotenoid

317   derivatives, (6S,7aR)-6-hydroxy-4,4,7a-trimethyl-6,7-dihydro-5H-1-benzofuran-2-one

318   (loliolide), (6S,7aS)-6-hydroxy-4,4,7a-trimethyl-6,7-dihydro-5H-1-benzofuran-2-one

319 (isololiolide), and 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-2(4H)-benzofuranone

320 (dihydroactinidiolide) were tentatively identified based on their mass fragmentation patterns and

321 retention times in GC-MS (Fig. 2C). As expected, dihydroactinidiolide eluted before isololiolide

322 and loliolide (Klok et al., 1984b), respectively, and were present in the same order in the

323 multidimensional GC×GC trace (Fig. 6). The GC×GC-TOF MS mass chromatogram for m/z

324 67, 111, 139, 154, 180, and 194 revealed not only these three compounds, but also two additional

325 carotenoid derivatives (Fig. S3), hexahydro-4,4,7a-trimethyl-2(3H)-benzofuranone

326 (tetrahydroactinidiolide), and 4,5,7,7a-tetrahydro-4,4,7a-trimethyl-2,6-benzofurandione

327 (dehydrololiolide).

328   Loliolide and isololiolide are known degradation products of fucoxanthin (Fig. S5, **III**), a

329 major carotenoid pigment that is found in diatoms (Klok et al., 1984a, 1984b; Repeta, 1989).

330 Though fucoxanthin can also be produced by dinoflagellates and haptophyte algae, both of which

331 have been observed in other Antarctic lakes (Coolen et al., 2004; Jaraula et al., 2009), their

332 occurrences have been shown to be proportional to the concentration of biogenic silica in some

333 lacustrine systems, making them robust proxies for diatom productivity (Castañeda et al., 2009,

334 2011). Fucoxanthin transformation into loliolide and isololiolide was hypothesized to be a

335 photooxidative process (Klok et al., 1984a, 1984b). The existence of a diagenetic pathway from

336 other carotenoids (i.e. β-carotene, zeaxanthin, or violaxanthin) to loliolide and isololiolide has

337 also been suggested (Isoe et al., 1972; Repeta, 1989). Repeta (1989) speculated that such

338 pathway from β-carotene may be associated to microbial activity, whereas a degradation

339 pathway from fucoxanthin is not microbially mediated.

340   Dihydroactinidiolide is part of the same structural family as loliolide, but is derived from

341 β-carotene (Fig. S5, **IV**; Isoe et al., 1972; Klok et al., 1984b; Kanasawud and Crouzet, 1990;

16

342   Gloria et al., 1993). Even though carotenes have been suggested as the biogenic precursors to

343   dihydroactinidiolide in anoxic marine sediments, an additional oxidation step mediated by

344   microbial fermentation from β-carotene to carotene epoxides is required before the molecule can

345   further degrade into lower molecular weight derivatives (Repeta, 1990). Dehydrololiolide, a

346   carotenoid-related compound (Uegaki et al., 1979), likely also came from the same source

347   pigment as loliolide, isololiolide, and dihydroactinidiolide, though the mechanism for its

348   formation is unclear. The tetrahydroactinidiolide, a saturated counterpart to dihydroactinidiolide,

349   is also tentatively assigned as a carotenoid-derivative in this study (Fig. S3).

350   Additionally, volatile carotenoid-derived compounds, 2,6,6-trimethyl-2-cyclohexene-1-4-

351   dione (ketoisophorone), 3,5,5-trimethyl-2-cyclohexenone (isophorone) and 2,2,6-trimethyl-1,4-

352   cyclohexanedione (dihydrooxophorone) observed in the UCM of the total DCM-extractable

353   environmental metabolome of LVBr (Fig. 2D), and 4-hydroxy-3,5,5-trimethyl-cyclohexenone

354   (4-hydroxyisophorone) detected via GC×GC-TOF MS (Fig. 7 and Fig. S4) have also been

355   previously attributed to the degradation products of β-carotene (Kanasawud and Crouzet, 1990).

356   The tentative identification of 4-ethynyl-4-hydroxy-3,5,5-trimethyl-2-cyclohexenone (Fig. S4)

357   raises the possibility that some of these carotenoid derivatives may have arisen from

358   diadinoxanthin (Fig. S5, **V**), bearing structural similarity to its side ring and functionality.

359   Diadinoxanthin is another phytoplankton carotenoid that have been used as a proxy for diatom

360   production in the lacustrine and marine habitats of East Antarctica (Verleyen et al., 2004).

361   Carotenoids have been extensively documented in the MDVs lakes of Antarctica, and are

362   used as a proxy for phytoplankton populations and diversity (Lizotte and Priscu, 1992, 1994;

363   Fritsen and Priscu, 1998; Squier et al., 2005). Most perennially ice-covered lakes are comprised

364   of microorganisms, dominated mostly by bacteria, algae, and heterotrophic protists (Fritsen and

365 Priscu, 1998; Morgan-Kiss et al., 2006; Bielewicz et al., 2011). Given the low temperature and

366 slow metabolic rates in LVBr and the fact that modern microbes are not photosynthetic, the

367 carotenoid derived compounds in the DCM-extractable fraction of LVBr environmental

368 metabolome are most likely the degradation products of legacy pigments that were produced in

369 the lake under previous environmental conditions, prior to the evaporation and encapsulation of

370 the residual brine. Whether the carotenoid derivatives observed in LVBr are exclusively formed

371 in a past ecosystem (as a result of photooxidation or autooxidation) or if they also represent, in

372 part, the metabolic breakdown of carotenoids by modern LVBr microbes, remains unclear.

373

374

375 *3.4 Implications for environmental metabolomics of Antarctic ecosystems*

376   For now, the presence of legacy compounds in the DCM-extractable environmental

377 metabolome of LVBr has challenged our ability to obtain an unambiguous signal of metabolites

378 from which we can infer the current metabolic activities. This challenge arises from the need to

379 assign compounds as being derived from the modern ecosystem, from legacy, or from both. The

380 examples of compounds shown here (chlorophyll and carotenoid derivatives) are easily assigned

381 to legacy in LVBr. Such a determination is not trivial for the hundreds of other compounds of the

382 brine, many of which remain to be identified. However, we have yet to analyze the particulate

383 microbial biomass material collected by filtration to look at metabolites associated with the

384 modern brine microbes.

385   In lacustrine sediments, organic carbon can be sourced autochthonously or

386 allochthonously (see review by Meyers and Ishiwatari, 1993). Source-specific refractory organic

387 compounds (biomarkers) can contain valuable information on the characteristics of past or

388 present environmental conditions as well as influences from detrital organic carbon input. The

389 contemporaneous contribution of organic material from different ages can usually be resolved by

390 compound-specific isotope analyses (Eglinton et al., 1996). Radiocarbon measurements of

391 aquatic organic carbon are calibrated based on the reservoir age, which is reflected by the mixing

392 of deep waters with surface layers that are in contact with the atmosphere. This conversion is not

393 trivial in systems like LVBr (past or present) since the "reservoir effect" are known to be highly

394 variable in the MDV lakes (Doran et al., 1999, 2014). Though the radiocarbon assessment of the

395 LVBr DOC provided constraints on the ages of the various fractions of LVBr DOC (Cawley et

396 al., 2016), paleometabolites such as those discussed in this study may serve as a practical

397 indicator for the legacy contribution of previous ecosystems into the total organic carbon pool

398 without using radiocarbon dating. Thus, compounds such as monopyrolles (deriving from

399 chlorophylls; Suzuki and Shioi, 1999) or norisoprenoids (deriving from carotenoids;

400 Winterhalter and Rouseff, 2002) are not only valuable in elucidating their source organism, but

401 may also retain information on the past environmental conditions that accompanied their

402 transformation in the evolving ecosystem.

403   Why is the legacy effect so prominent in LVBr? The presence of a legacy component in

404 the metabolites of LVBr is concordant with the high DOC level and low-temperature limited

405 microbial community. Significant alteration of the DOC reservoir is unlikely because of the

406 temperature-limited microbial growth rates, with an estimated generation time of ~120 years

407 (estimated by leucine incorporation into proteins; Murray et al., 2012). This slow metabolism is

408 reflected in the very abundant DOC of LVBr (580 mg-C·L^{-1}; Cawley et al. 2016) compared to

409 e.g. the Blood Falls' subglacial brine in Taylor Valley, Antarctica, with a DOC of 9.25 mg-C·L^{-1}

410 for a generation time of about ~300 days (estimated by thymidine incorporation into DNA;

411   Mikucki et al., 2004, 2009). Characterization of LVBr dissolved organic matter demonstrated

412   that the brine's DOC inventory is composed of a combination of compounds deriving from

413   modern microbial processes as well as a dominant, altered legacy component (Cawley et al.,

414   2016).

415   Ecological legacies in Antarctica have been extensively described as a functional link

416   between past and present ecosystems, which ultimately influences the variations in biological

417   activities overtime (McKnight et al., 1999; Moorhead et al., 1999; Knoepfle et al., 2009). In

418   perennially ice-covered lakes, stratification can persist over long periods of time and pools of

419   nutrients can collect in significant concentrations in deeper waters (Priscu et al., 1999). For

420   example, the bottom waters of the west lobe of Lake Bonney (Taylor Valley, MDVs) have an

421   apparent radiocarbon age of $22,950 \pm 250$ years BP, totally disconnected from the modern

422   processes occurring in the upper water column (Doran et al., 2014). The dry valley lakes appear

423   to contain legacies from past ecosystem conditions that have captured a record of evolving

424   limnology. In LVBr, the legacy component of metabolites are dominated by compounds derived

425   from photosynthetic algae and bacteria, even though the current ecosystem is entirely dominated

426   by non-photosynthetic bacteria.

427   Some significant information on the past ecosystem is retained in paleometabolites and

428   may provide constraints on our understanding of Lake Vida's past biogeochemical processes.

429   The presence of maleimides derived from chlorophyll *a* suggests that the previous lake's

430   ecosystem was supported by primary productivity, whereas the occurrence of maleimides that

431   originated from bacteriochlorophyll *c*, *d*, or *e*, biomarkers for green sulfur bacteria, suggests that

432   at one point, the lower water column of Lake Vida's was euxinic (anoxic and sulfidic) and that

433 the euxinic water penetrated into the photic zone, or the conditions permitted light to penetrate to

434 the benthos which was supported with euxinic conditions.

435   The occurrence of carotenoid derivatives in LVBr is an effect of legacy, as the

436 carotenoids themselves were likely synthesized during previous environmental conditions and

437 were photooxidized or autooxidized prior to the brine encapsulation. Though it is likely that the

438 catabolism of these compounds occurred in the prior ecosystem as well, we cannot discount the

439 possibility that the modern LVBr microbes are actually degrading or have degraded those

440 carotenoids.

441

442

443 *3.5 Paleometabolites in other slow-growing ecosystems*

444   Our research points to a question of whether legacy significantly affects the interpretation

445 of data obtained on organic material in other slow-growing ecosystems. In a habitat that has very

446 low metabolic activities, and consequently slow turnover rates, the legacy metabolites would be

447 very slowly, or not at all, degraded.

448   The conditions of permafrost cryopegs such as temperature and salinity as well as cell

449 counts are comparable to those of LVBr (Gilichinsky et al., 2003; Murray et al., 2012;

450 Colangelo-Lillis et al., 2016). Biological activities of cryopeg communitites inferred from

451 reazurin reduction rate (Bakermans et al., 2003) and ^{14}C-labelled glucose uptake (Gilichinsky et

452 al., 2003) have been demonstrated in the laboratory at temperatures as low as -10°C and -15°C,

453 respectively (Gilichinsky et al., 2003). Considering the low temperatures of the cryopegs,

454 extremely slow *in situ* metabolic rates are likely, thereby limiting biological activities to

455 maintenance of vital cell functions for survival (i.e. DNA, amino acid racemization, and cell

456   membrane repair) as suggested by Gilichinsky et al. (2003). Thus, it is likely that cryopeg brines

457   contain legacy metabolites. To the best of our knowledge, no data on the constituents or age of

458   organic matter of cryopeg communities have been reported. Obtaining such information may

459   help reveal the extent at which legacy metabolites co-occur with modern metabolites from extant

460   communities with slow metabolic rates.

461   The global deep subsurface contains a microbial biosphere with cell numbers estimated to

462   reach up to ~10^{30}, with cell densities averaging ~5×10^5 cells/mL in the subsurface marine

463   sediments (below 1 km) compared to ~5×10^8 cells/mL in near-surface (0-10 cm) marine

464   sediments (Whitman et al., 1998). In the deep subsurface (continental and marine), cells have

465   extremely slow generation times due to energy-limited conditions (Chapelle et al., 1988;

466   D'Hondt et al., 2002; Jørgensen and Boetius, 2007). Previous attempts to estimate the extant

467   microbial biomass and to characterize the diversity of microorganisms in the deep subsurface

468   utilize a wide array of methods such as viable cell count (Cragg et al., 1992; Chapelle et al.,

469   2002), direct cell staining (Cragg et al., 1992; Chapelle et al., 2002; Schippers et al., 2005), as

470   well as genomic-based (Chapelle et al., 2002; Schippers et al., 2005) and lipid-based analyses

471   (Harvey et al., 1986; Sturt et al., 2004; Lipp et al., 2008). Many of these analyses rely on the

472   detection and quantification of organic material, which abundance depends on the fluxes of

473   metabolites being synthesized or degraded by the extant community. In the deep subsurface, the

474   energy limitation has a significant control on the speed of metabolic activities (Hoehler and

475   Jørgensen, 2013). We suggest that the legacy components of these deep subsurface environments

476   can persist for long periods of time and may significantly convolute data interpretation of *in situ*

477   organic material obtained from the deep subsurface.

478

4. Conclusions

Many of the compounds discovered in the DCM-extractable environmental metabolome of LVBr can be traced to degraded photosynthetic pigments (chlorophylls and carotenoids). The paleometabolites in the DCM-extractable metabolome of LVBr are derived from previous environmental conditions and do not represent biological activities of the modern bacterial community. This dominance in legacy components is concordant with the subzero environmental temperatures and slow metabolisms of LVBr microbes. Consequently, the information on the extant community carried by the total dissolved organic carbon pool can be mixed with legacy signals, and in the case of LVBr, can be difficult to detect due to low signal amongst a large legacy "noise". As a result, interpretation of exogenous materials contemporaneous to ecosystem production with low metabolic rates is difficult, as the large pool of legacy compounds may mask the biological signals of the extant microbial community. However, the presence of legacy materials is an opportunity to observe paleometabolites that represent past biogeochemical processes. Ultimately, the presence of a legacy component in slow and isolated microbial ecosystems is a caveat that must first be addressed in order to prevent the misinterpretation of the current metabolite collection in the total dissolved organic carbon pool. Furthermore, in order to characterize the current metabolites, extant ecosystem biomass also needs to be assessed.

507

508 **Figures**

509

510

Figure 1: (A) Map of Antarctica showing the location of the McMurdo Dry Valleys. (B) Satellite

image of Victoria Valley showing the location and outline of Lake Vida (taken by Landsat 7 on

December 18, 1999). The yellow square indicates the borehole from which brine was sampled

during the 2010 expedition. (C) Cross-section schematic of Lake Vida showing the location of

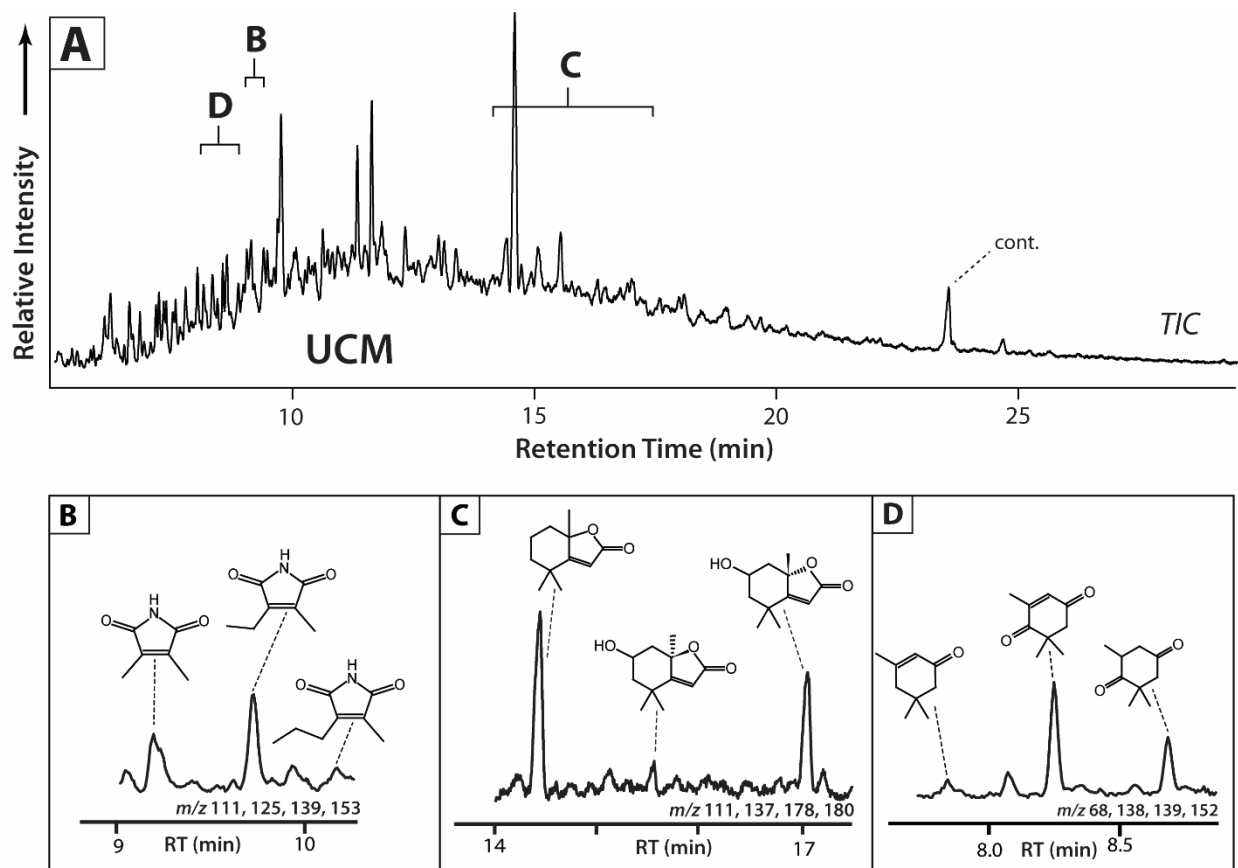the interstitial brine underneath the frozen lake body and the >10 cm sediment layers.

Figure 2: (A) GC-MS total ion chromatogram (TIC) of LVBr showing unresolved complex mixture (UCM). "Cont." = common lab contaminant. (B) summed ion chromatogram m/z 111, 125, 139, 153, major fragmentation and molecular ions for Me,Me, Me,Et and Me,Pr maleimides. (B) summed ion chromatogram m/z 111, 137, 178, and 180, major fragmentation and molecular ions for loliolide, isololiolide, and dihydroactinidiolide. (C) summed ion chromatogram m/z 68, 138, 139, and 152, major fragmentation and molecular ions for isophorone, ketoisophorone and dihydroketoisophorone. Note that the summed ion chromatograms are not presented in the order of retention time.
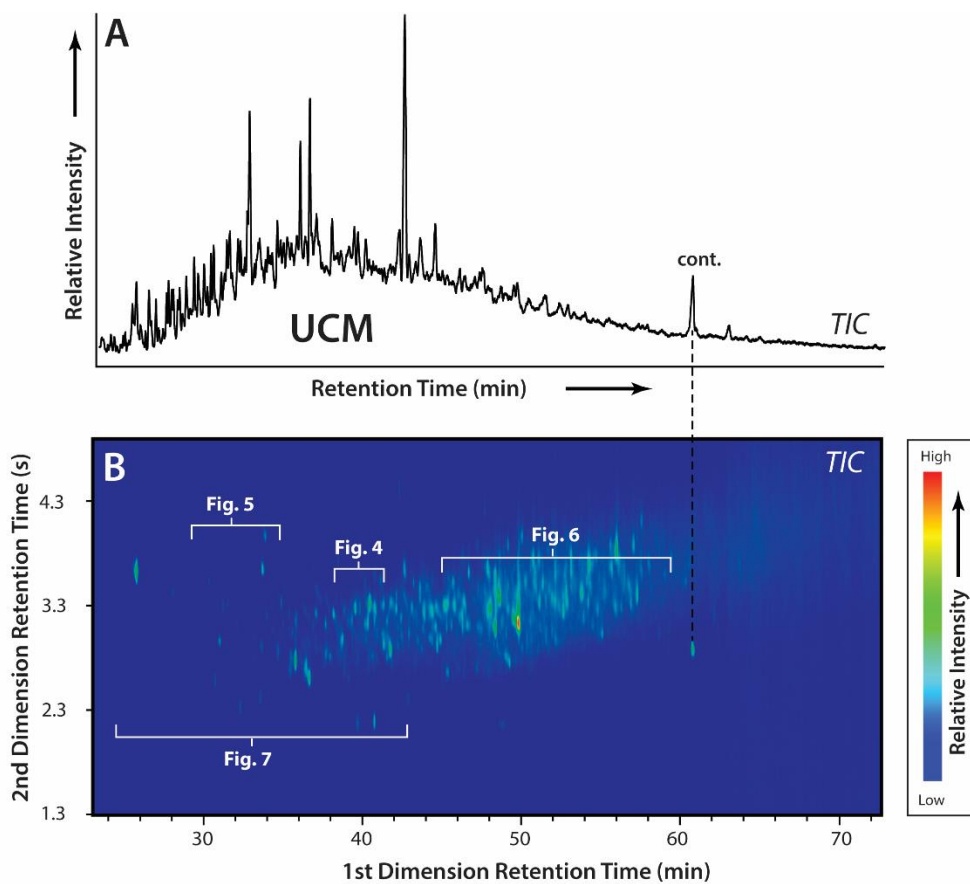
Figure 3: (A) GC-MS TIC of Lake Vida brine total DCM-extractable environmental

metabolome containing UCM. "Cont." = common lab contaminant. (B) GC×GC-TOF MS TIC
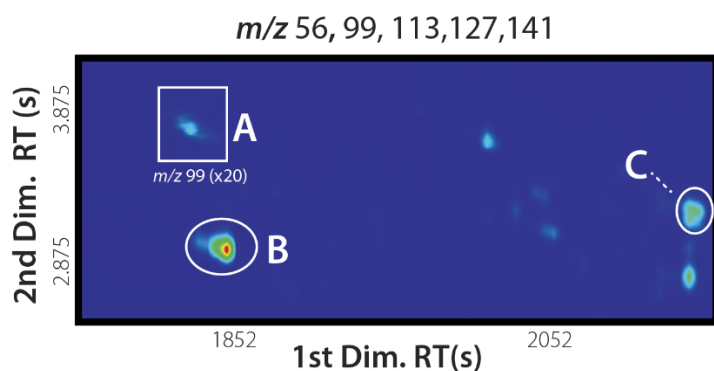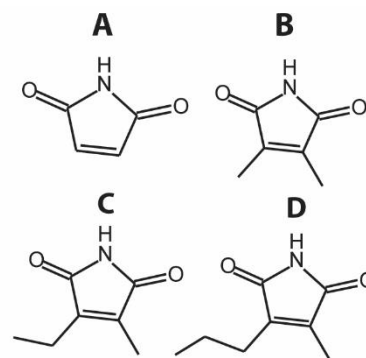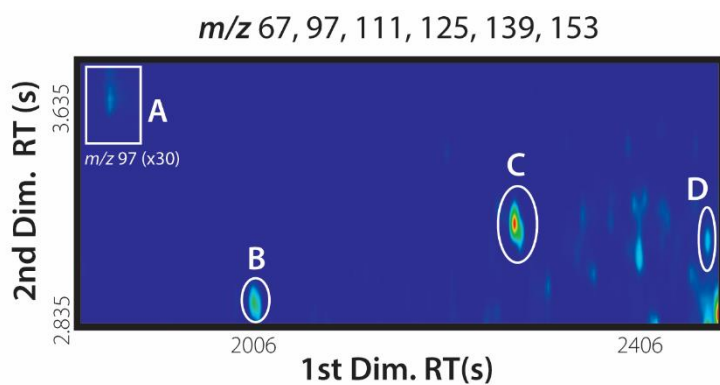
of the same sample.
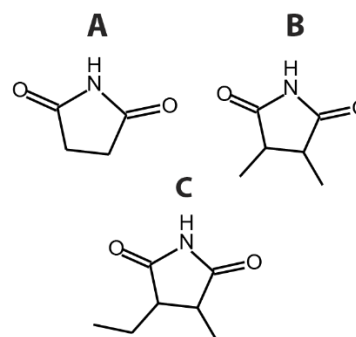
Figure 4: GC×GC-TOF MS of summed mass chromatogram for major fragment (*m/z* 67) and molecular ions (*m/z* 97, 111, 125, 139, and 153) for maleimides.

Figure 5: GC×GC-TOF MS of summed mass chromatogram for major fragment (*m/z* 56) and molecular ions (*m/z* 99 (x20 intensity), 113, 127, 141) for succinimides.
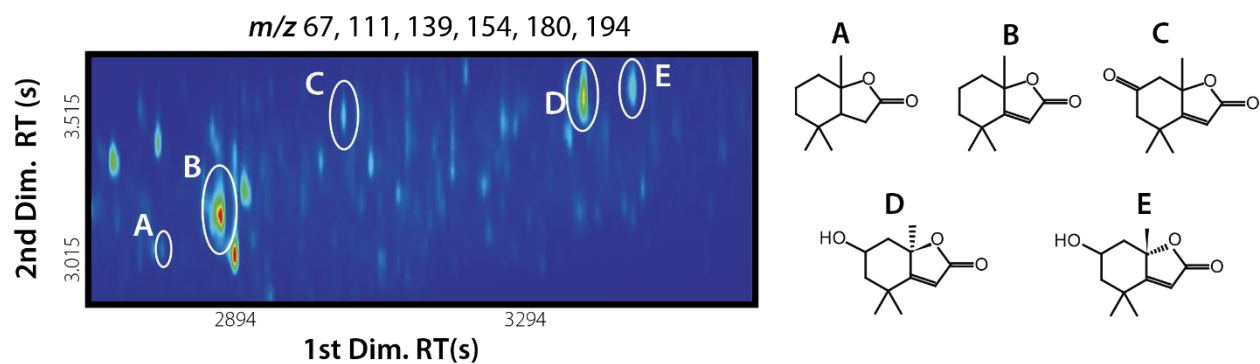
Figure 6: GC×GC-TOF MS of summed mass chromatogram for major fragment ions of tetrahydroactinidiolide, dihydroactinidiolide, dehydrololiolide, isololiolide, and loliolide.
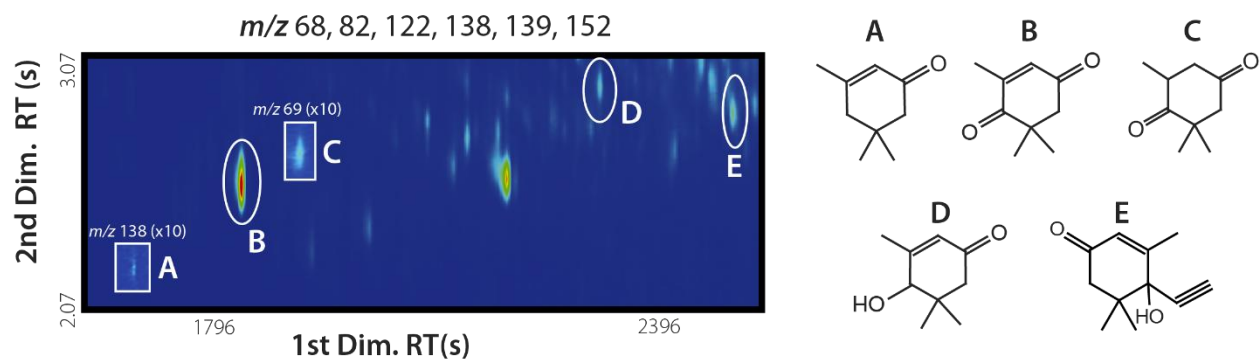

Figure 7: GC×GC-TOF MS of summed mass chromatogram for major fragment ions of volatile carotenoid-derivatives.

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