

1 **Title:** Ecological theory applied to environmental metabolomes reveals compositional  
2 divergence despite conserved molecular properties

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26 **Abstract**

27 Stream and river systems transport and process substantial amounts of dissolved organic matter  
28 (DOM) from terrestrial and aquatic sources to the ocean, with global biogeochemical  
29 implications. However, the underlying mechanisms affecting the spatiotemporal organization of  
30 DOM composition are under-investigated. To understand the principles governing DOM  
31 composition, we leverage the recently proposed synthesis of metacommunity ecology and  
32 metabolomics, termed ‘meta-metabolome ecology.’ Applying this novel approach to a freshwater  
33 ecosystem, we demonstrated that despite similar molecular properties across metabolomes,  
34 metabolite identity significantly diverged due to environmental filtering. We refer to this  
35 phenomenon as ‘thermodynamic redundancy,’ which is analogous to the ecological concept of  
36 functional redundancy. We suggest that under thermodynamic redundancy, divergent  
37 metabolomes can support equivalent biogeochemical function just as divergent ecological  
38 communities can support equivalent ecosystem function. As these analyses are performed in  
39 additional ecosystems, potentially generalizable principles, like thermodynamic redundancy, can  
40 be revealed and provide insight into DOM dynamics.

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## 49 **Introduction**

50 Riverine ecosystems receive substantial carbon inputs from terrestrial sources ( $\sim 1.9 \text{ Pg C yr}^{-1}$ ),  
51 releasing some into the atmosphere and transporting a large portion to the ocean ( $\sim 0.95 \text{ Pg C yr}^{-1}$ ).  
52 Much of this carbon is dissolved and complexed with other elements as organic matter. As  
53 this dissolved organic matter (DOM) travels through watersheds (e.g., along river corridors), it  
54 interacts with resident microbial communities and undergoes significant biochemical  
55 transformations that influence its fate<sup>1,3-7</sup>. Recent research has suggested that these ongoing  
56 biochemical reactions have a significant influence on river corridor biogeochemistry<sup>5,6,8</sup>. Despite  
57 the significance of these DOM biochemical reactions, predictive models (e.g., Earth system  
58 models, reactive transport models) generally do not represent these detailed processes because  
59 they are largely unknown<sup>4,6</sup>. Moreover, the underlying principles governing the detailed  
60 chemistry of DOM are under-investigated<sup>5</sup>. Our capacity to predict changes in the functioning of  
61 coupled terrestrial-aquatic systems (e.g., watersheds) will be enhanced by resolving these  
62 uncertainties<sup>3,7,9</sup>.

63  
64 Recent studies have continued to elucidate principles governing riverine DOM processing<sup>5,6,10</sup>.  
65 Graham *et al.* 2017<sup>10</sup> revealed that microorganisms within riverbed sediments preferentially  
66 targeted organic molecules based on their thermodynamic favorability, thereby deterministically  
67 altering DOM chemistry. Stegen *et al.* 2018<sup>5</sup> further demonstrated that hyporheic zone  
68 metabolism was governed by mixing effects which removed thermodynamic protection (i.e., a  
69 “priming effect”). Accordingly, Graham *et al.* 2018<sup>6</sup> demonstrated that DOM chemistry better  
70 predicted microbial respiration rates than community composition, metabolic potential, or  
71 expressed metabolisms. Together, these studies indicate a strong connection between DOM

72 chemistry and realized biogeochemical function, and that deterministic processes underlie  
73 spatiotemporal variation in DOM chemistry.

74

75 The recently proposed synthesis of meta-community ecology and metabolomics, termed “meta-  
76 metabolome ecology,” provides new opportunities to deepen understanding of the processes  
77 governing DOM chemistry<sup>11</sup>. This framework treats organic molecules in the environment as  
78 ‘ecosystem metabolites’ that are both resources for and products of microbial metabolism. A  
79 given DOM pool can therefore be thought of as an assemblage of ecosystem metabolites  
80 analogous to ecological communities. The framework further suggests that studying the  
81 contributions of different ecological assembly processes can offer novel interpretations with  
82 biogeochemical implications<sup>11</sup>. To operationalize the conceptual framework, ecological null  
83 models can be applied to metabolite assemblages to quantify the relative influences of  
84 deterministic and stochastic processes governing metabolome dynamics.

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86 Understanding the relative contributions of deterministic and stochastic processes can help reveal  
87 mechanisms driving differences in the molecular properties of DOM pools<sup>11</sup>. Deterministic  
88 processes<sup>12</sup> result from forces in the environment that systematically change the probabilities of  
89 observing a given metabolite. This can occur by changing the rates that a given metabolite is  
90 produced or transformed, which is analogous to ecological selection changing the birth or death  
91 rate of a given biological species. In context of the metabolite assemblages comprising DOM  
92 pools, deterministic processes are therefore the outcome of the environment selecting for or  
93 against a given metabolite. In contrast, stochastic processes<sup>12</sup> are the result of random events that  
94 lead to uncoordinated increases or decreases in prevalence of individual metabolites.

95 Stochasticity can arise through uncoordinated changes in rates of production or transformation  
96 (analogous to random birth/death events in ecological systems) as well as via non-selective  
97 transport (analogous to dispersal in ecological systems). Stochasticity dominates when  
98 deterministic processes (e.g., selective agents) are not applied consistently through space and/or  
99 time, or are too weak to overcome factors such as spatial mixing of metabolites<sup>12-14</sup>.

100

101 Further analogies can be drawn to ecological systems whereby stochastic and deterministic  
102 processes can be separated into different classes to deepen understanding of the forces governing  
103 the molecular properties of metabolite assemblages<sup>11</sup>. As in ecological systems, the influences of  
104 deterministic processes can separate into variable and homogenous selection. Variable selection  
105 occurs when selective pressures cause assemblages that are separated in space or time to diverge  
106 in composition. In turn, differences in metabolite composition are greater than would be  
107 expected by random chance<sup>12,13</sup>. In contrast, homogenous selection occurs when selective  
108 pressures cause assemblages to have similar composition; differences in metabolite composition  
109 are less than expected by random chance<sup>12,13</sup>. A dominant influence of stochastic processes  
110 results in differences in metabolite composition that do not deviate from a random expectation<sup>15</sup>.  
111 While stochastic processes can also be separated into two classes<sup>12,13</sup>, doing so is beyond the  
112 scope of the current study.

113

114 Linking the relative influences of variable selection, homogenous selection, and stochastic  
115 processes to system dynamics (i.e., hydrology, geochemistry) provides opportunities to better  
116 understand spatiotemporal dynamics of metabolite assemblages and inform the representation of  
117 DOM chemistry in predictive models. A primary analytical challenge is quantifying the relative

118 influences of deterministic and stochastic processes. As shown in Danczak *et al.*<sup>11</sup>, this challenge  
119 can be overcome using metabolite null modeling, which borrows directly from ecological null  
120 modeling through the use of dendrograms representing biochemical relationships among  
121 metabolites. In ecological systems, null models are often based on phylogenetic and/or functional  
122 trait relationships (e.g., Swenson *et al.* 2012<sup>16</sup>, Siefert *et al.* 2013<sup>17</sup>, and Dini-Andreote *et al.*  
123 2015<sup>14</sup>). Using metabolite null modeling, Danczak *et al.*<sup>11</sup> found that biochemical relationships  
124 among metabolites can strongly influence spatial variation in river corridor metabolite  
125 assemblages. This points to an opportunity to leverage metabolite null modeling to reveal new  
126 principles governing the molecular properties of metabolite assemblages comprising DOM.

127  
128 Here we use concepts (e.g., stochastic/deterministic processes) and analytical tools (e.g., null  
129 models) derived from community ecology to investigate fundamental aspects of metabolite  
130 assemblages with respect to (1) within and among assemblage diversity (i.e., alpha and beta  
131 diversity), (2) stochastic and deterministic processes governing assemblage composition, and (3)  
132 the relationship between stochastic and deterministic processes and metabolite chemistry (e.g.,  
133 thermodynamic properties and elemental composition). For this, we study the temporal dynamics  
134 of both stream and streambed pore water from a low-order river corridor within the HJ Andrews  
135 Experimental Forest which has long been the focus of river corridor research<sup>18-21</sup>. This system is  
136 representative of steep, low-order river corridors, which dominate headwater river networks both  
137 in terms of abundance and relative drainage area<sup>22</sup> and where riverbed (e.g., hyporheic zone)  
138 biogeochemical processes can dominate total respiration<sup>23-25</sup>. We find that despite very similar  
139 molecular and thermodynamic properties in bulk DOM pools given by high resolution mass  
140 spectrometry (i.e., elemental composition, double-bond equivalent, etc.), deterministic processes

141 drove divergence in the biochemical transformations connecting metabolites, both between and  
142 within surface and pore waters. Furthermore, our results point to a new concept referred to as  
143 ‘thermodynamic redundancy’ in which spatially or temporally separate metabolite assemblages  
144 have indistinguishable thermodynamic properties despite divergence in other metabolome  
145 characteristics.

146

## 147 **Results**

148 **Metabolite properties were similar across surface and pore water.** Given that the sampled  
149 surface water had likely passed through the subsurface multiple times within the studied field  
150 system<sup>21,26,27</sup>, we expected metabolite assemblages within the surface and pore water to share  
151 some molecular properties. This was borne out with respect to properties inferred directly from  
152 assigned molecular formulae. More specifically, the surface and pore water metabolite  
153 assemblages had similar thermodynamic and molecular properties (**Figure 2**). The standard  
154 Gibb’s Free Energy of carbon oxidation ( $\Delta G^{\circ}_{\text{cox}}$ ), double-bond equivalents (DBE), and modified  
155 aromaticity index ( $AI_{\text{Mod}}$ ) did not significantly differ between surface and pore water (p-value >  
156 0.05). While the thermodynamic and molecular properties varied through time, they did not  
157 clearly follow diel hydrological dynamics (**Figure 1**). Similarities in thermodynamic and  
158 molecular properties between surface and pore water may be due to significant hydrologic  
159 connectivity in the study system<sup>19–21</sup>. This mixing has the potential to minimize the signatures of  
160 organic matter processing within surface or subsurface domains. Follow-on analyses reveal that  
161 mixing does not, however, fully overcome the signatures of localized processes (as discussed  
162 below).

163

164 **Conserved alpha diversity and molecular properties contrast with divergence in**  
165 **composition, revealing thermodynamic redundancy.** Additional analyses examining both  
166 within metabolome diversity (i.e., alpha diversity) and among metabolome differences in  
167 composition (i.e., beta diversity) presented an apparent contradiction; metabolomes with similar  
168 within-metabolome properties and diversity had divergent composition. This leads to the  
169 proposed concept of thermodynamic redundancy, discussed below. More specifically, the  
170 dendrogram-based alpha diversity values were largely similar between surface and subsurface  
171 metabolomes mirroring dynamics in molecular and thermodynamic properties (**Figure 3**).  
172 Patterns of Faith's PD mostly followed molecular property patterns, indicating that there were no  
173 major differences in dendrogram structure between surface and pore water metabolomes (p-  
174 value: 0.063). Other alpha diversity metrics that use dendrogram-based relational information  
175 (i.e., MPD, MNTD, VNTD, VPD) followed similar trends between surface and pore water  
176 metabolomes (p-value: > 0.1). These results indicate that across surface and porewater there are  
177 conserved molecular properties and biochemical transformation network topologies, both of  
178 which are used to estimate the dendrogram used for alpha diversity analyses. Alpha diversity  
179 analyses do not, however, directly evaluate variation in composition across metabolomes. Beta-  
180 diversity metrics can be used to make such comparisons.

181  
182 Comparison of metabolome assemblages using beta diversity metrics revealed significant  
183 divergence in metabolome composition, despite the high degree of similarity in alpha diversity.  
184 More specifically, Jaccard dissimilarity and  $\beta$ -mean nearest taxon distance ( $\beta$ MNTD) principal  
185 coordinate analysis (PCoA) plots showed clear separation between surface and subsurface  
186 metabolomes (**Figure 4**; Jaccard p-value – 0.005;  $\beta$ MNTD p-value – 0.02). Furthermore, the

187 Jaccard-based analyses reveal significantly greater differences than did  $\beta$ MNTD. This reflects  
188 patterns observed within the dendrograms used in the estimation of  $\beta$ MNTD, but not used to  
189 estimate Jaccard; similarities in molecular properties were captured in the dendrogram resulting  
190 in decreased separation across the  $\beta$ MNTD ordination, relative to the Jaccard-based PCoA.  
191 Taken together, these results demonstrate that metabolite profiles with indistinguishable  
192 molecular and thermodynamic characteristics, as well as similar levels of alpha diversity, can  
193 nonetheless be composed of different metabolites when viewed at the level of specific  
194 metabolites. This opens the possibility that localized—and potentially temporally variable—  
195 deterministic processes drive spatiotemporal variation in metabolite assemblages, which  
196 ultimately result in habitat-specific metabolomes.

197

198 Results discussed above present an apparent contradiction whereby there is divergence in  
199 composition, but consistency in thermodynamic/molecular properties and alpha-diversity  
200 metrics. To reconcile these outcomes, we propose the concept of thermodynamic redundancy.  
201 Conceptually, thermodynamic redundancy is similar to the ecological observation of functional  
202 redundancy, whereby different biological taxa can fill the same functional role. In the case of  
203 thermodynamic redundancy, different metabolite assemblages are comprised of different  
204 metabolites (analogous to biological taxa) but have similar thermodynamic and molecular  
205 properties. Given strong influences of DOM thermodynamics in river corridors, we propose the  
206 hypothesis that thermodynamic properties of individual metabolites are analogous to functional  
207 roles of biological taxa.

208

209 From an ecological perspective, functional redundancy has been observed repeatedly in both  
210 microbial communities (with respect to metagenomic profiles) and macro-organisms such as  
211 plant communities (with respect to functional traits such as specific leaf area)<sup>28-31</sup>. For example,  
212 the human gut can have numerous different steady state microbial communities that all exhibit  
213 healthy function due to redundant metabolisms<sup>32</sup>. We hypothesize that this analogy extends to  
214 metabolites in that different assemblages may support the same biogeochemical function (e.g.,  
215 net rate of denitrification) by meeting some given thermodynamic requirements. Alternatively,  
216 thermodynamic redundancy may instead capture the biogeochemically-relevant historical  
217 processes that led to metabolomes with similar molecular properties but divergent composition,  
218 rather than true functional diversity.

219

220 The degree to which thermodynamic redundancy is observed across metabolite assemblages will  
221 require data from a broad suite of environmental systems. It will be important to evaluate this  
222 concept with paired measured biogeochemical rates and with more detailed metabolome data that  
223 include information on molecular structure to assess its impact on the potential functional role of  
224 organic metabolites. Regardless of the degree to which thermodynamic redundancy indicates true  
225 functional redundancy, extending the general concept of redundancy to metabolomes further  
226 emphasizes the significant breadth of conceptual parallels between ecological communities and  
227 metabolite assemblages.

228

### 229 **Divergence in metabolite assemblages was associated with biochemical transformations.**

230 The concept of thermodynamic redundancy indicates conserved thermodynamic properties  
231 despite strong divergence in metabolite composition. Through additional multivariate analyses

232 we found that this divergence was driven by transformations that were used to define  
233 biochemical relationships among metabolites in our analyses. More specifically, through a  
234 Jaccard dissimilarity-based NMDS analysis we found that profiles of biochemical  
235 transformations were divergent between surface and subsurface metabolomes (**Figure 5**; p-value:  
236 0.0082). Examining the transformations by elemental composition showed that transformations  
237 containing only C, H, and O were significantly more frequent within surface water (p-value:  
238 0.014) while N-containing transformations (including the loss or gain of amino acids) occurred  
239 more frequently within the pore water (p-value: 0.008). Previous work has also shown greater  
240 abundance of N-based transformations in pore water, relative to surface water<sup>5</sup>. While we can  
241 only speculate, these results suggest that generalizable principles might exist in terms of how  
242 biochemical transformations vary between surface and pore water.

243  
244 As in Stegen *et al.*<sup>5</sup>, we suggest that the subsurface has a greater capacity for biomass turnover  
245 and proteolytic activity due to increased microbial load as compared to the surface water. We  
246 also suggest that the higher frequency of N-transformations in the pore water were not due to  
247 differences in N limitation causing enhanced N mining given that N concentrations (e.g., NO<sub>3</sub>,  
248 NO<sub>2</sub>, and total N) were below our limit for detection in both surface and pore water  
249 (**Supplemental File 1**). However, we did not measure organic N, so we cannot exclude the  
250 possibility that N was more limited in the subsurface than the surface due to potentially greater  
251 microbial load. Alternatively, these differences could arise from hotspot activity which has been  
252 reported within other riverbed sediments/hyporheic zones<sup>6</sup>. Regardless of the mechanism, the  
253 consistency between this study and previous work suggests that shallow subsurface domains  
254 (often associated with hyporheic zones) may consistently be characterized by greater abundance

255 of N-containing biogeochemical transformations. Multi-system comparative studies will be  
256 needed to evaluate this possibility, which could emerge as a principle that is transferable across  
257 river corridor systems, providing an opportunity to inform the structure of mechanistic predictive  
258 models.

259

260 **Deterministic processes drove differences between surface and pore water metabolite**

261 **assemblages.** Divergence in metabolite assemblage composition through space or time can be

262 due to stochastic processes, deterministic processes, or some combination of the two.

263 Deterministic processes can have strong influences when biotic or abiotic features cause

264 systematic differences in organismal reproductive success or metabolite expression across

265 assemblages<sup>15</sup>. Stochastic processes can arise due to spatiotemporal differences whereby random

266 or uncoordinated ‘demographic events’ (i.e., organismal birth/death or metabolite

267 expression/transformation) lead to divergence in composition that is not due to systematically

268 imposed deterministic factors<sup>13,33</sup>. Stochastic processes can also be dominant when there is

269 significant movement or mixing of organisms/metabolites across spatial locations (i.e., across

270 ecological communities or metabolite assemblages). The  $\beta$ -nearest taxon index ( $\beta$ NTI) metric, a

271 phylogenetic null modeling approach, has been shown to quantitatively estimate the relative

272 contributions of these stochastic and deterministic processes<sup>12,13,15</sup>. This provides much deeper

273 insight into the mechanisms driving observed spatiotemporal patterns in community/assemblage

274 composition when compared to more traditional methods such as ordinations, redundancy

275 analysis, or regressions.

276

277 Applying null modeling approaches to metabolite assemblages showed that divergences  
278 observed through ordination analysis (**Figure 3**) were overwhelmingly due to deterministic  
279 processes that arise from differences in abiotic and/or biotic features. Specifically, the  
280 deterministic processes observed here were akin to the concept of ‘variable selection’ in  
281 ecological communities. Variable selection can dominate the assembly of communities when  
282 features of the environment systematically drive divergence in composition by causing spatial or  
283 temporal shifts in the relative fitness of different biological taxa. We infer that an analog to  
284 variable selection driven by features in the biotic and/or abiotic environment is causing  
285 divergence in metabolite assemblages within our study system despite conserved levels of alpha-  
286 diversity and molecular properties (**Figures 2 and 3**). It is important to recognize that this is not  
287 a pre-determined outcome of sampling different locations within the river corridor. The  
288 divergence between surface and porewater metabolite assemblages could have been due to  
289 limited exchange enabling compositional divergence to arise through uncoordinated (i.e.,  
290 stochastic) changes in metabolite production and transformation. Such a scenario would have  
291 been akin to dispersal limitation enabling ecological drift, which is itself akin to genetic drift  
292 within the theory of population genetics<sup>34</sup>. Recent application of the  $\beta$ NTI null model to river  
293 corridor metabolite assemblages from the mainstem of the Columbia River showed that such  
294 stochastic scenarios are possible and potentially likely<sup>11</sup>.

295

296 Examining dynamics within surface or porewater revealed stronger influences of deterministic  
297 processes in porewater (relative to surface water), suggesting highly localized biotic or abiotic  
298 processes with very strong influences over assemblage composition. Furthermore, porewater  
299 metabolomes were more consistently governed by variable selection than those in surface water

300 **(Figure 6;** p-value: < 0.001). This was true despite the study system appearing to be well-mixed,  
301 whereby advective transport of water-soluble metabolites could overwhelm deterministic  
302 processes causing compositional divergence (akin to ‘mass effects’ in ecological meta-  
303 communities)<sup>13,35</sup>. Based on correlations with other physical and chemical variables,  
304 deterministic pressures within the surface water seem to be associated with geochemical  
305 conditions, including sulfate and dissolved oxygen concentrations (**Supplemental File 2**). No  
306 physical or chemical variables were significantly related to the level of determinism associated  
307 with porewater metabolite assemblages. These results suggest that different biogeochemical  
308 processes are at play in surface and subsurface domains, despite the surface water being an  
309 integration of pore water through space and time<sup>18-21</sup>.

310

311 One of the key biogeochemical differences between surface and subsurface domains in the study  
312 system and in other river corridors<sup>5</sup> is the variation of putative biochemical transformations. This  
313 inference is supported through analyses linking these putative biochemical transformations to  
314 influences of deterministic processes. The relative frequencies of many individual biochemical  
315 transformations, regardless of the molecule gained or lost, were significantly correlated to the  
316 level of determinism. For most transformations, these correlations were similar between surface  
317 and pore water metabolite assemblages (**Supplemental File 3**). Grouping transformations by  
318 elemental compositions as above, however, revealed that determinism in the surface water was  
319 positively associated with N-, S-, and P-containing transformations and negatively related to  
320 those transformations containing only C, H, and O. These results indicate that as N-, S-, and P-  
321 containing transformations become more frequent within the surface water, overall metabolome  
322 composition begins to diverge. Within the porewater, only S-containing transformations were

323 significantly positively related to deterministic processes. The absence of a strong N-containing  
324 transformation correlation within the porewater contrasts with the overall frequency dynamics  
325 discussed earlier and likely points to more complex organic N metabolism. To further reveal  
326 underlying processes and their dynamics will require more detailed geochemical (e.g., dynamics  
327 of vertical redox gradients) and molecular investigations (e.g., metatranscriptomics of microbial  
328 communities), likely across other river corridors and longer time periods.

329

### 330 **Discussion**

331 A key element limiting accurate representation of DOM cycling in predictive models is  
332 understanding the processes governing spatiotemporal variation in metabolite assemblages and  
333 the follow-on impacts to emergent biogeochemical function. To address this challenge, we took a  
334 novel approach based on concepts and methods from metacommunity ecology. We find that  
335 deterministic processes drive divergence in metabolite assemblage composition through both  
336 space and time. This divergence was observed despite similar alpha diversity and  
337 molecular/thermodynamic properties. We also provide evidence that deterministic processes  
338 which cause metabolome divergence are associated with organic transformations. This indicates  
339 that expressed microbial metabolisms should be highly dynamic in time and should diverge  
340 between surface and subsurface components of the river corridor. Given strong similarity in  
341 molecular properties across surface and subsurface domains, we further propose that divergent  
342 metabolite assemblages have the potential to be thermodynamically equivalent.

343

344 This highlights a major, unresolved question that is fundamental to understanding the role of  
345 environmental metabolites—as both reactants and products—in emergent biogeochemical

346 function. That is, what are the processes that give rise to observed metabolite assemblages and  
347 what is the interplay of these processes with biogeochemical function? Future work should focus  
348 on understanding the degree to which variation in the composition of metabolite assemblages  
349 influences variation in biogeochemistry irrespective of changes in molecular properties. This is  
350 analogous to the question of how important microbial community composition is to realized  
351 biogeochemical function<sup>6,36,37</sup>. It is often found that microbial composition itself is not a primary  
352 driver of biogeochemical function, which indicates a significant amount of functional  
353 redundancy<sup>6,38,39</sup>. In other cases, however, microbial community composition corresponds well  
354 with ongoing biogeochemical processes. For example, arsenic mobilization within contaminated  
355 soils in Bangladesh was driven by the presence and distribution of diverse taxa associated with  
356 arsenic and iron reducing bacteria<sup>40</sup>.

357

358 Similar functional profiles despite divergent taxonomic composition is a common feature in  
359 ecological systems<sup>31,37,41</sup>. Different microbial communities within the human gut or in soil  
360 environments will provide similar (if not indistinguishable) contributions to overall ecosystem  
361 function<sup>32</sup>. Analogously, divergent metabolite assemblages can have indistinguishable  
362 thermodynamic and molecular properties, though this does not necessarily indicate that the  
363 metabolite assemblages are identical with respect to biogeochemical function. Both the surface  
364 and pore water metabolite assemblages had conserved thermodynamic and molecular properties  
365 but were compositionally divergent due to strong deterministic processes (**Figure 6**). This  
366 suggests that compositionally divergent metabolite assemblages could be redundant with respect  
367 to bulk biogeochemical processes (e.g., respiration rates) that have been shown to be influenced  
368 by metabolite thermodynamics<sup>6,42</sup>. Whether these outcomes are driven by differential substrate

369 preference across the riverbed or common labile carbon depletion, the divergence in metabolite  
370 assemblages suggests that these environments can take different paths while maintaining similar  
371 bulk chemical and thermodynamic properties. In other words, redundancy appears to exist at  
372 higher levels of metabolite properties, but not at the lower levels associated with biochemical  
373 linkages among metabolites. An open question is the degree to which net biogeochemical rates  
374 respond to higher-level properties (e.g., thermodynamics of individual metabolites) versus lower-  
375 level biochemical mechanisms. Evaluating this question is fundamental to understanding  
376 whether and how thermodynamic redundancy is association with redundancy of biogeochemical  
377 function.

378

379 Metabolite assemblages are examined as snapshots of the organic compounds at a given point in  
380 time and space. By analyzing assemblages together and viewing them as analogs to ecological  
381 communities, we can draw upon the concepts, theory, and tools developed with meta-community  
382 ecology. Doing so provides novel insight into the processes that shape spatiotemporal dynamics  
383 of metabolite assemblages. Here, using this approach we found that variable selection can  
384 dominate spatial and temporal dynamics of metabolite assemblages, potentially via underlying  
385 biochemical processes associated with dynamic organic N, S, and P metabolism. Similarities  
386 between this study and previous work hint at the potential to elucidate generalizable principles  
387 that could be used to enhance the predictive capacity of process-based simulation models (e.g.,  
388 reactive transport codes). Applying our analytical framework to ecosystem metabolomes from a  
389 broad suite of river corridors and pairing these analyses with biogeochemical rate measurements  
390 will provide exciting opportunities to test and reveal generalizable principles.

391

## 392 **Methods**

393 *Site Description.* Samples for this study were collected from Watershed 1 (WS01) in the HJ  
394 Andrews Experimental Forest, Oregon, USA (**Figure 1**)<sup>19,21</sup>. For a detailed description of this  
395 study site, please refer to Ward *et al.*<sup>21</sup> and Wondzell *et al.*<sup>19</sup>. Briefly, WS01 is a shallow, low-  
396 order, headwater stream which is hydrologically connected to the surrounding terrestrial  
397 environment<sup>19,21</sup>. The river corridor is forested, and evapo-transpiration drives diel fluctuations  
398 in stream discharge (**Figure 1**)<sup>26,27</sup>. Given that these hydrologic dynamics occur with regular  
399 frequency, they offer an opportunity to study changes in DOM composition through time in both  
400 the surface water and pore water. This study was conducted under low-discharge conditions  
401 during July 23-25, 2018, when diel stage fluctuations can cause spatially intermittent flows, the  
402 proportion of total down valley flow passing through the hyporheic zone is maximized, and  
403 connectivity between the subsurface and surface was the highest. Therefore, the surface water  
404 collected at the sampling location has likely passed through the hyporheic zone multiple  
405 times<sup>21,26,27</sup>.

406

407 *Sample Collection.* Three points separated by ~4 meters were selected along the river corridor to  
408 collect pore water samples. Approximately 20 mL of pore water was collected from each of these  
409 locations every 3 hours for 48 hours. Concurrently, surface water was collected in triplicate from  
410 the same spatial position as the central pore water location. In total, 102 total samples were  
411 collected over 17 time points. Surface water was collected using a 60 mL syringe through Teflon  
412 tubing while the pore water was collected using a syringe attached via Teflon tubing to a 30 cm  
413 long stainless-steel sampling tube (MHE Products, MI, USA) with a slotted screen across the  
414 bottom ~5cm. One sampling tube was installed to 30cm depth at each pore water sampling

415 location; these tubes remained in place during the 48-hour time course of sampling. Prior to  
416 sampling a given location, the syringe was flushed 3 times with the source water to ensure only  
417 the desired water was collected. All samples were filtered through a 0.2  $\mu\text{m}$  Sterivex filter  
418 (Millipore, MA, USA). At each time point, one filter was used for all pore water samples, and a  
419 different filter was used for the surface water. To minimize contamination, water passing through  
420 a given filter was collected for analysis using a needle attached to the filter and injected through  
421 a septum. During sampling, water temperature, approximate water stage, and pH were measured.  
422 Water samples for DOM analysis were injected into amber borosilicate glass vials. Samples for  
423 cations and anions were injected into clear borosilicate glass vials. Once collected, samples were  
424 stored in a cooler on blue ice until they could be frozen until they were processed in the lab.

425  
426 *Geochemistry.* Anion concentrations were measured using a Dionex ICS-2000 anion  
427 chromatograph with AS40 autosampler using an isocratic method (guard column: IonPac AG18  
428 guard, 4x50mm; analytical column: IonPac AS18, 4x250mm; suppressor: RFIC ASRS, 300  
429 rmm, self-regenerating; suppressor current: 57mA). The isocratic method was a 15-minute run  
430 with a 1 mL/min flow rate with 22 mM KOH at 30 degrees C and 25  $\mu\text{L}$  injection volume.  
431 Standards were made from Spex CertiPrep (Metuchen, NJ, 08840) 1000 mg/L anion standards.  
432 NO<sub>2</sub> standard was diluted in the range of 0.04 to 20 ppm. F standard was diluted in the range of  
433 0.2 to 10 ppm. Cl and SO<sub>4</sub> standards were diluted in the range of 0.16 to 80 ppm. NO<sub>3</sub> standard  
434 was diluted in the range of 0.12 to 60 ppm. Ion peaks were identified and integrated manually in  
435 the software.

436

437 Cations samples were prepared with nitric acid. Samples were measured with a Perkin Elmer  
438 Optima 2100 DV ICP-OES with an AS93 auto sampler. A Helix Tracey 4300 DV spray chamber  
439 and SeaSpray nebulizer were used with double distilled 2 % nitric acid (GFS Chemicals, Inc.  
440 Cat. 621) and a flow rate of 1.5 mL/min. Calibration standards were made with Ultra Scientific  
441 ICP standards (Kingstown, RI). P, Mg, Ca, K, and Na standards were diluted in the range of 5-  
442 4000 ppm. Fe standard was diluted in the range of 0.5-400 ppm.

443

444 Non-purgeable organic carbon (NPOC) was determined by a Shimadzu combustion carbon  
445 analyzer TOC-L CSH/CSN E100V with ASI-L auto sampler. An aliquot of sample was acidified  
446 with 15% by volume of 2N ultra-pure HCL. The acidified sample was then sparged with carrier  
447 gas for 5 minutes to remove the inorganic carbon component. The sparged sample was injected  
448 into the TOC-L furnace at 680°C using 100 uL injection volumes. The best 3 out of 4 injections  
449 replicates were averaged to get final result. The NPOC organic carbon standard was made from  
450 potassium hydrogen phthalate (Nacalia Tesque, lot M7M4380). The calibration range was 0.5 to  
451 10 ppm NPOC as C.

452

453 *Fourier Transform Ion Cyclotron Resonance Mass Spectrometry Sample Preparation, Data*  
454 *Collection, and Data Preprocessing.* Fourier Transform Ion Cyclotron Resonance Mass  
455 Spectrometry (FTICR-MS) was used to provide ultrahigh resolution characterization of  
456 metabolite assemblages within each DOM sample. Aqueous samples (NPOC 0.33-0.99 mg C/L)  
457 were acidified to pH 2 with 85% phosphoric acid and extracted with PPL cartridges (Bond Elut),  
458 following Dittmar *et al.*<sup>43</sup>. Subsequently, high-resolution mass spectra of the organic matter were  
459 collected using a 12 Tesla (12T) Bruker Solarix Fourier transform ion cyclotron resonance mass

460 spectrometer (Bruker, Solarix, Billerica, MA) located at the Environmental Molecular Sciences  
461 Laboratory in Richland, WA. Samples were directly injected into the instrument using a custom  
462 automated direct infusion cart that performed two offline blanks between each sample. The  
463 FTICR-MS was outfitted with a standard electrospray ionization (ESI) source, and data was  
464 acquired in negative mode with the needle voltage set to +4.4kV, resolution was 220K at  
465 481.185 m/z. Data were collected with an ion accumulation time of 0.08 sec and 0.1 sec from  
466 100 m/z – 900 m/z at 4M. One hundred forty-four scans were co-added for each sample and  
467 internally calibrated using OM homologous series separated by 14 Da (–CH<sub>2</sub> groups). The mass  
468 measurement accuracy was typically within 1 ppm for singly charged ions across a broad m/z  
469 range (100 m/z - 900 m/z). BrukerDaltonik Data Analysis (version 4.2) was used to convert raw  
470 spectra to a list of m/z values by applying the FTMS peak picker module with a signal-to-noise  
471 ratio (S/N) threshold set to 7 and absolute intensity threshold to the default value of 100.  
472 Chemical formulae were assigned using Formularity<sup>44</sup>, an in-house software, following the  
473 Compound Identification Algorithm<sup>45–47</sup>. Chemical formulae were assigned based on the  
474 following criteria: S/N >7, and mass measurement error < 0.5 ppm, taking into consideration the  
475 presence of C, H, O, N, S and P and excluding other elements. This in-house software was also  
476 used to align peaks with a 0.5 ppm threshold.

477  
478 The R package ftmsRanalysis<sup>48</sup> was then used to remove peaks that either were outside the  
479 desired m/z range (200 m/z – 900 m/z) or had an isotopic signature, calculate a number of  
480 derived statistics (Kendrick defect, double-bond equivalent, aromaticity index, nominal  
481 oxidation state of carbon, standard Gibb’s Free Energy of carbon oxidation), and organize the  
482 data into a common framework<sup>49–52</sup>. Samples that were run at both ion accumulation times were  
483 combined; given that different IATs will detect different compounds<sup>53</sup>, by combining the two

484 IATs we can gain a more complete characterization of the metabolite assemblages. Replicates  
485 were further combined such that if a metabolite was present in one replicate, it was included in  
486 the composite assemblage. Because peak intensities cannot be used to infer concentration, all  
487 peak intensities were changed to binary presence/absence. In turn, observing a metabolite in  
488 multiple replicates was equivalent to observing it in a single replicate; the absence of a peak is  
489 defined as below the limit of detection. One sample (PP48\_000012) was considered an outlier

490

491 *Metabolite Dendrogram Estimation.* A transformation-weighted characteristics dendrogram  
492 (TWCD) was generated following the protocol outlined in Danczak *et al.*<sup>11</sup>. First, biochemical  
493 transformations were identified within the dataset according to the procedure employed by  
494 Breitling *et al.*<sup>54</sup>, Bailey *et al.*<sup>55</sup>, Graham *et al.*<sup>6,10</sup>, and Garayburu-Caruso *et al.*<sup>42</sup>. The pairwise  
495 mass differences between each detected metabolite were determined and matched to a database  
496 of 1298 frequently observed biochemical transformations (**Supplemental File 4**). For example,  
497 if the mass difference between two metabolites was 18.0343, that would putatively indicate a  
498 loss or gain of an ammonium group, while a mass difference of 103.0092 would putatively  
499 indicate loss or gain of a cysteine. This calculation is enabled by the ultrahigh mass resolution of  
500 FTICR-MS data; given this resolution, we considered any between-metabolite mass difference  
501 within 1 ppm of the expected mass of a transformation to be a match. This analysis provides two  
502 outputs: a transformation profile outlining the number of times a putative transformation could  
503 occur in a given sample and pairwise mass difference between every peak. Multivariate  
504 similarities between the transformation profiles of each sample were visualized by generating a  
505 Jaccard dissimilarity-based non-metric multidimensional scaling (NMDS) ordination (*metaMDS*,  
506 ‘vegan’ package v2.5-6)<sup>56</sup>. Using these pairwise mass differences and transformation

507 associations, we then generated a transformation network in which nodes are metabolites and  
508 edges are transformations (**Supplemental Figure 1**)<sup>11,57,58</sup>. Relationships between metabolites  
509 were determined by first selecting the largest cluster of interconnected nodes (discarding  
510 everything not within this cluster) and measuring the stepwise distance between each pair of  
511 metabolites (i.e., the minimum number of transformations required to connect one metabolite to  
512 another metabolite within the largest cluster of the biochemical transformation network). These  
513 pairwise distances were then standardized between 0 and 1.

514  
515 Relationships among metabolites were also evaluated using a number of metabolite  
516 characteristics estimated from inferred molecular formulae. To do so elemental composition (C-,  
517 H-, O-, N-, S-, P-content), double-bond equivalents (DBE), modified aromaticity index ( $AI_{mod}$ ),  
518 and Kendrick's defect were used as metabolite characteristics indicating molecular composition  
519 and structure of the metabolites. These metrics were combined to generate a pairwise Euclidean  
520 distance matrix with each distance representing approximate dissimilarity (i.e., further distances  
521 indicate less similar metabolites). These molecular differences were then weighted by the  
522 previously measured transformation distances that were themselves scaled to be between 0 and 1.  
523 A UPGMA hierarchical clustering analysis was then used to convert this combined distance  
524 matrix into a dendrogram which approximates the relationships among metabolites  
525 (**Supplemental File 5**). This resulted in the transformation weighted molecular characteristics  
526 dendrogram (TWCD). While Danczak *et al.*<sup>11</sup> used three different dendrograms, doing so is  
527 beyond the scope of the current study and we chose to use the TWCD as it integrates more  
528 information relative to other dendrogram methods.

529

530 *Diversity Analyses.* The metabolite data were treated as an assemblage of ecological units  
531 following the methodology outlined in Danczak *et al.*<sup>11</sup>. All metabolites were treated on a  
532 presence/absence basis – peak intensities were not used due to charge competition<sup>47,52</sup>. Richness  
533 measurements and Jaccard-based dissimilarity metrics (*vegdist*, ‘vegan’ package 2.5-6)<sup>56</sup> were  
534 used to assess the compositional differences among metabolite assemblages. The TWCD was  
535 used to measure dendrogram-based alpha-diversity indices including Faith’s PD (*pd*, ‘picante’  
536 package v1.8)<sup>59</sup>, mean nearest taxon distance (MNTD), mean pairwise distance (MPD), variance  
537 in nearest taxon distance (VNTD), and variance in pairwise distance (VPD) (*generic.metrics*,  
538 ‘pez’ package v1.2-0)<sup>60-65</sup>.  $\beta$ -mean nearest taxon distance ( $\beta$ MNTD) was measured using the  
539 *comdistnt* function in the picante R package<sup>59</sup>. Jaccard dissimilarity and  $\beta$ MNTD results were  
540 visualized using a principal coordinates analysis (PCoA; *pcoa*, ‘ape’ package v5.3)<sup>66</sup>.

541  
542 *Ecological Null Modeling.* Null modeling was performed to quantify the relative influences of  
543 variable selection, homogeneous selection, and stochastic processes over metabolite  
544 assemblages<sup>11</sup>. Specifically, the  $\beta$ -Nearest Taxon Index ( $\beta$ NTI) was calculated to measure the  
545 influence of stochastic and deterministic assembly processes<sup>12,13,15</sup>.  $\beta$ NTI was estimated for each  
546 pairwise assemblage comparison. To do so, a null distribution of 999  $\beta$ MNTD values were  
547 generated and compared to the observed  $\beta$ MNTD value for a given pair of assemblages. Pairwise  
548 comparisons with  $|\beta$ NTI| > 2 indicate that deterministic processes were responsible for observed  
549 differences in metabolite composition. In contrast, pairwise comparisons with  $|\beta$ NTI| < 2 indicate  
550 that stochastic processes were responsible for observed differences in metabolite composition.

551

552 Furthermore, the deterministic processes can be separated into two classes. When  $\beta\text{NTI} > 2$ ,  
553 differences in metabolite composition are greater than would be expected by random chance (i.e.,  
554 greater than the stochastic expectation). This is analogous to ‘variable selection,’ which occurs  
555 when deterministic processes drive divergence in composition between a pair of  
556 assemblages<sup>13,14</sup>. When  $\beta\text{NTI} < 2$ , differences in metabolite composition are less than the  
557 stochastic expectation. This is analogous to ‘homogeneous selection,’ which occurs when  
558 deterministic process drive convergence in composition between a pair of assemblages. Mean  
559  $\beta\text{NTI}$  values for each sample were obtained and used in all analyses and plots.

560

561 *Statistics and Plot Generation.* Differences in distributions (i.e., diversity analyses, molecular  
562 properties) were evaluated using Mann Whitney U tests (*wilcox.test*, ‘stats’ package).  
563 Multivariate differences (i.e., ordinations) were identified using PERMANOVA tests (*adonis*,  
564 *vegan* package v2.5-6)<sup>56</sup>. All correlations were Spearman-based and were performed using the  
565 *rcorr* function (‘Hmisc’ package v4.2)<sup>67</sup>. All boxplots and scatter/line plots were generated using  
566 the ‘ggplot2’ R package (v3.2.1)<sup>68</sup>; three-dimensional ordinations were generated using the  
567 ‘plot3D’ R package (v1.1.1)<sup>69</sup>.

568

569 All R scripts used within this manuscript are available on GitHub at  
570 <https://github.com/danczakre/HJA-FTICR-Ecology>. The uncalibrated, peak-picked FTICR-MS  
571 files and aqueous geochemistry data are available at [https://data.ess-](https://data.ess-dive.lbl.gov/view/doi:10.15485/150969570)  
572 [dive.lbl.gov/view/doi:10.15485/150969570](https://data.ess-dive.lbl.gov/view/doi:10.15485/150969570). The FTICR-MS report used in this study has been  
573 included as **Supplemental Data 1**.

574

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743

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752

#### 753 **Author Contributions**

754 R.E.D, A.E.G., E.B.G, and J.C.S. conceptualized the study. V.A.G., J.W.M., L.R., and J.R.W.  
755 collected samples and analyzed anions/cations. R.K.C, J.G.T., and N.K collected FTICR-MS  
756 data and assisted with analyses. S.P.H. and A.S.W. assisted with hydrological interpretations.  
757 R.E.D. performed the ecological and statistical analyses. R.E.D. drafted the manuscript but all  
758 authors contributed to the writing

759

## 760 **Competing Interests**

761 The authors declare no competing financial interests.

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## 766 **Figure Legends**

767 Figure 1: An outline of the study site and associated hydrology. a) Map of Watershed 1 within  
768 the HJ Andrews Experimental Forest in Oregon. b) Hydrograph for Watershed 1 with the  
769 sampling period highlighted in red and expanded upon in the inset. Sampling points are indicated  
770 by the blue dashed lines in the inset.

771

772 Figure 2: Plots of average chemical properties through time, separated by environment (i.e., pore  
773 water and surface water). Abbreviations are as follows - standard Gibb's Free Energy of carbon  
774 oxidation ( $\Delta G^{\circ}_{\text{cox}}$ ), modified aromaticity index ( $AI_{\text{Mod}}$ ), and double-bond equivalents (DBE).  
775 Peak counts refer to the number of peaks within a given sample.

776

777 Figure 3: Boxplots illustrating metabolome alpha diversity. Abbreviations are as follows –  
778 Faith's Phylogenetic Diversity (PD), species richness (SR), mean pairwise distance (MPD),  
779 mean nearest taxon distance (MNTD), variation of pairwise distance (VPD), and variation in  
780 nearest taxonomic distance (VNTD). If a p-value is listed, significant differences were identified  
781 using a Mann Whitney U test.

782

783 Figure 4: Metabolome beta diversity principal coordinate analyses (PCoA). a) Jaccard  
784 dissimilarity-based PCoA b)  $\beta$ MNTD-based PCoA. Significant differences between groups (i.e.,  
785 pore water and surface water) were determined using PERMANOVA and are indicated in the  
786 bottom graph.

787

788 Figure 5: An investigation of potential biochemical transformations throughout the watershed. a)  
789 Jaccard-based non-metric multidimensional scaling (NMDS) graph for transformation profiles,  
790 with significant differences between groups determined by PERMANOVA and indicated in the  
791 bottom right. b) Boxplots comparing the relative proportion of transformations with specific  
792 elemental compositions observed within pore or surface water. Significance indicated by Mann  
793 Whitney U tests are indicated in the bottom or top left. For example, the surface water had a  
794 significantly higher proportion of transformations containing only C, H, and O than the pore  
795 water.

796

797 Figure 6:  $\beta$ -nearest taxon index ( $\beta$ NTI) calculations across the watershed. a) Boxplots illustrating  
798 differences in  $\beta$ NTI results. Mann Whitney U test significance is indicated in the upper right  
799 corner. b) Mean  $\beta$ NTI for each time point separated by water type.

800

## 801 **Supplemental Legend**

802 Supplemental Figure 1: Visual representation of the transformation network utilized to generate  
803 the transformation-weighted characteristics dendrogram (TWCD). Each node within the network

804 represents an individual metabolite while the edges connecting each node is a transformation.

805 Note the large cluster of interconnected nodes near the middle of the plot.

806

807 Supplemental File 1: Metadata and geochemistry for the field site at Watershed 1 (WS1) in the

808 HJ Andrews Experimental Forest.

809

810 Supplemental File 2: Significant Spearman-based correlations between average sample  $\beta$ NTI and

811 site geochemistry. The table is short given that only significant correlations are provided.

812 Correlations labeled “Bulk” indicate that both surface water and pore water samples were

813 considered in correlations (i.e., the entire dataset), correlations labeled “SW48” were performed

814 only with surface water samples, and correlations labeled “PP48” were performed only using

815 pore water samples.

816

817 Supplemental File 3: Significant correlations between average sample  $\beta$ NTI and putative

818 biochemical transformations. Sheet 1 includes those significant correlations between individual

819 transformation relative proportions and  $\beta$ NTI, while Sheet 2 are all correlations between

820 transformation groups and  $\beta$ NTI (i.e., not only significant correlations). As in Supplemental File

821 2, correlations labeled “Bulk” indicate that both surface water and pore water samples were

822 considered in correlations (i.e., the entire dataset), correlations labeled “SW48” were performed

823 only with surface water samples, and correlations labeled “PP48” were performed only using

824 pore water samples.

825

826 Supplemental File 4: Database of known and frequently observed biochemical transformations.

827 This file is used to identify putative biochemical transformations using ultrahigh-resolution mass

828 differences obtained from FTICR-MS datasets.

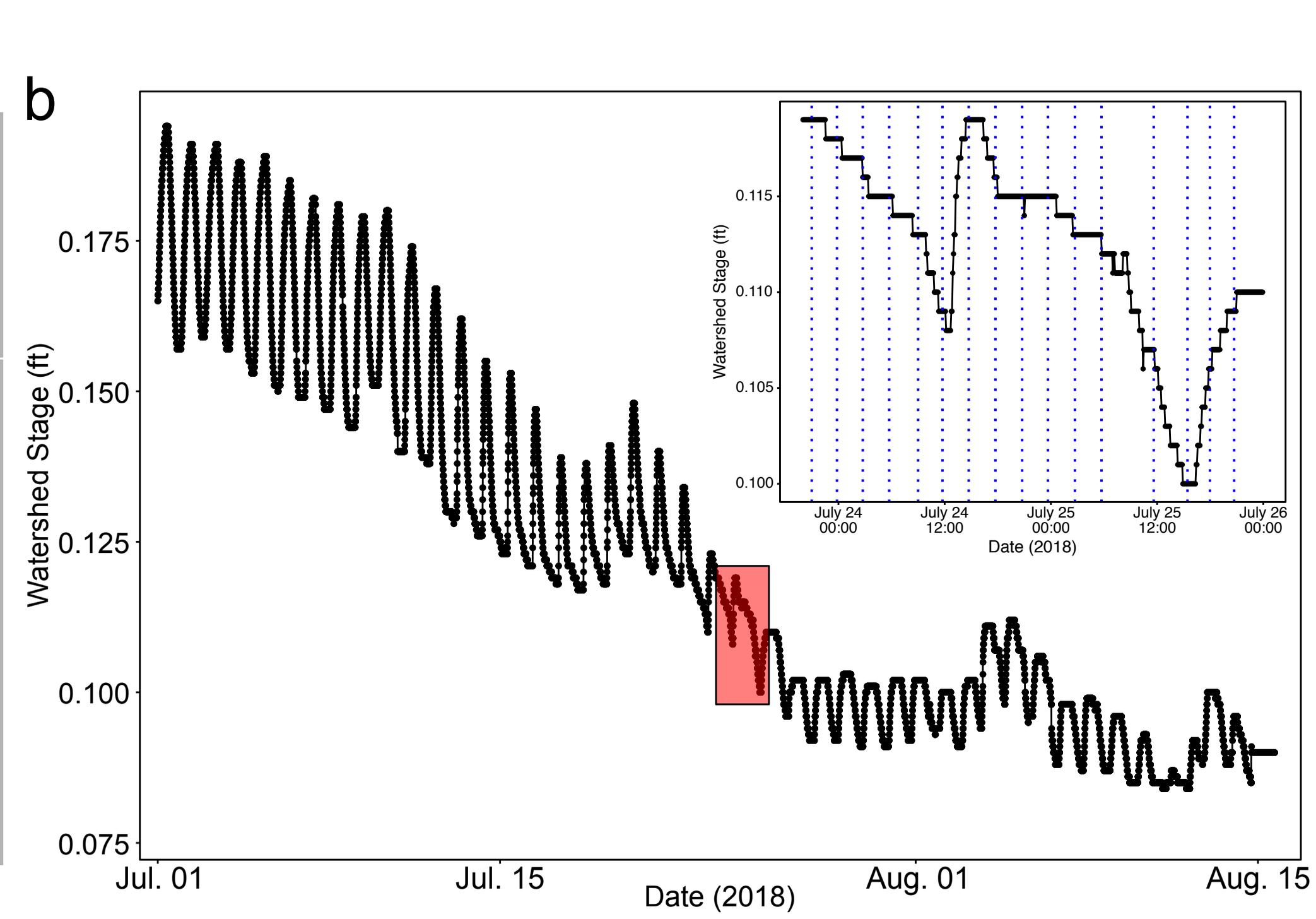
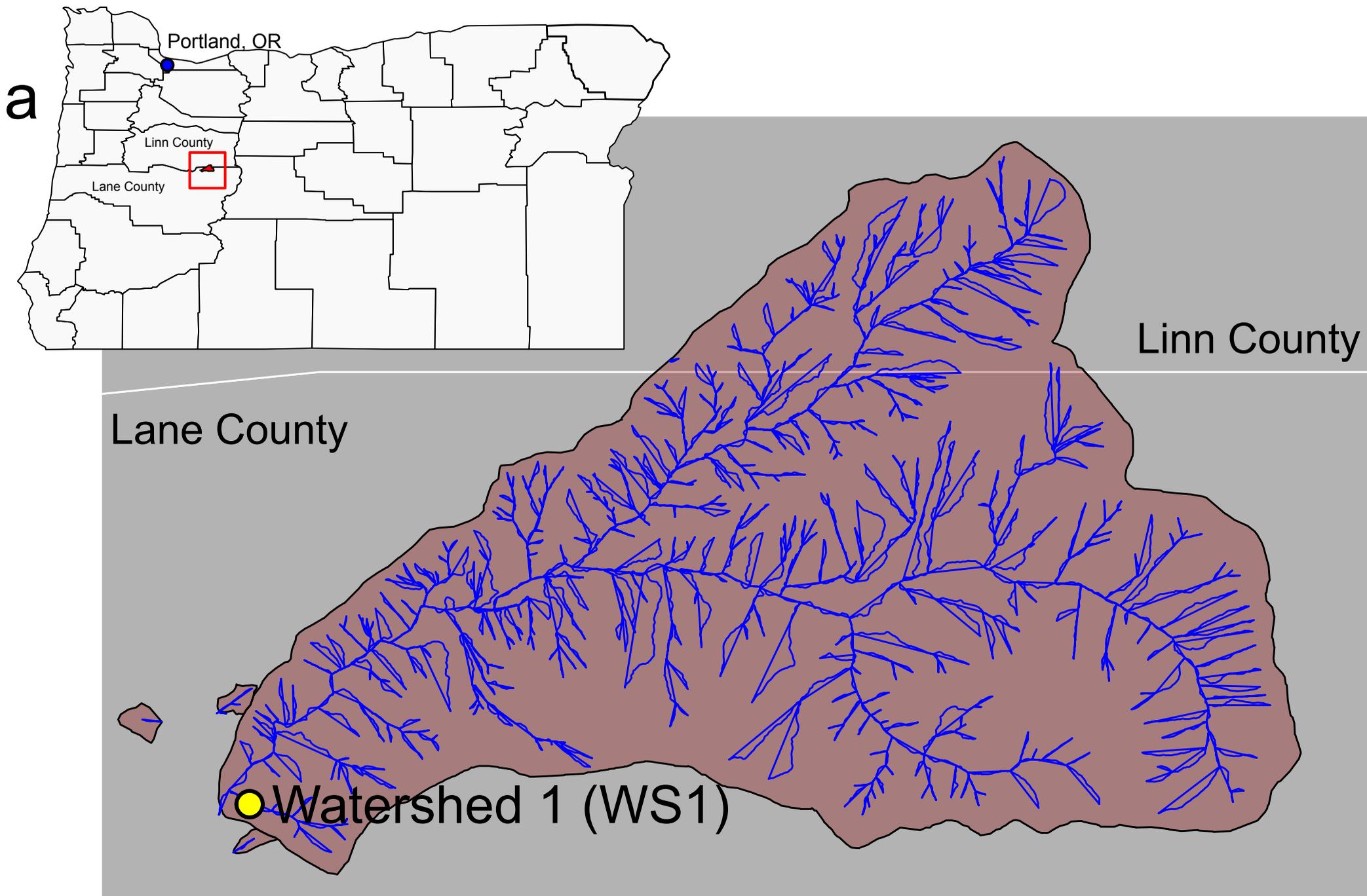
829

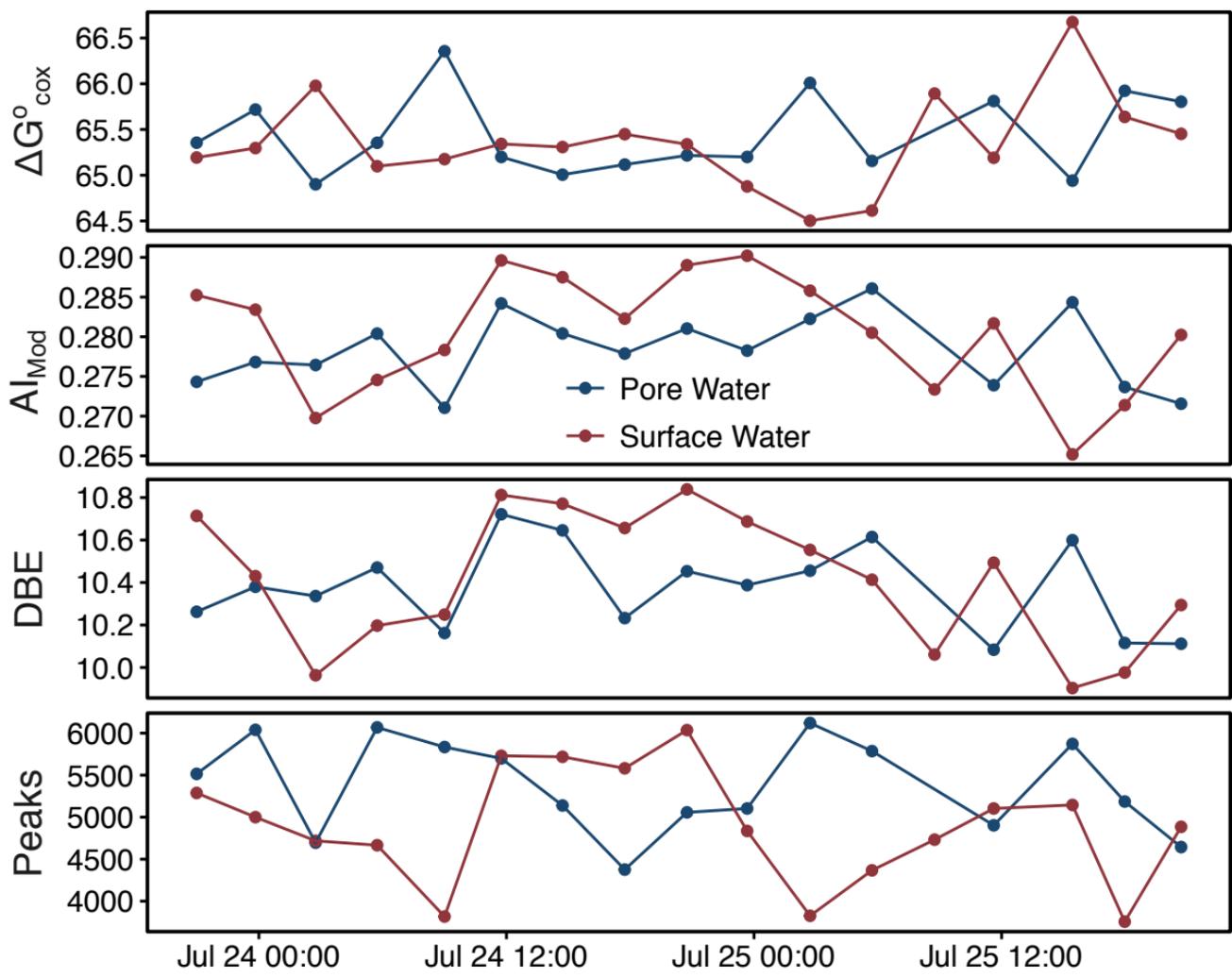
830 Supplemental File 5: The transformation-weighted characteristics dendrogram (TWCD) obtained

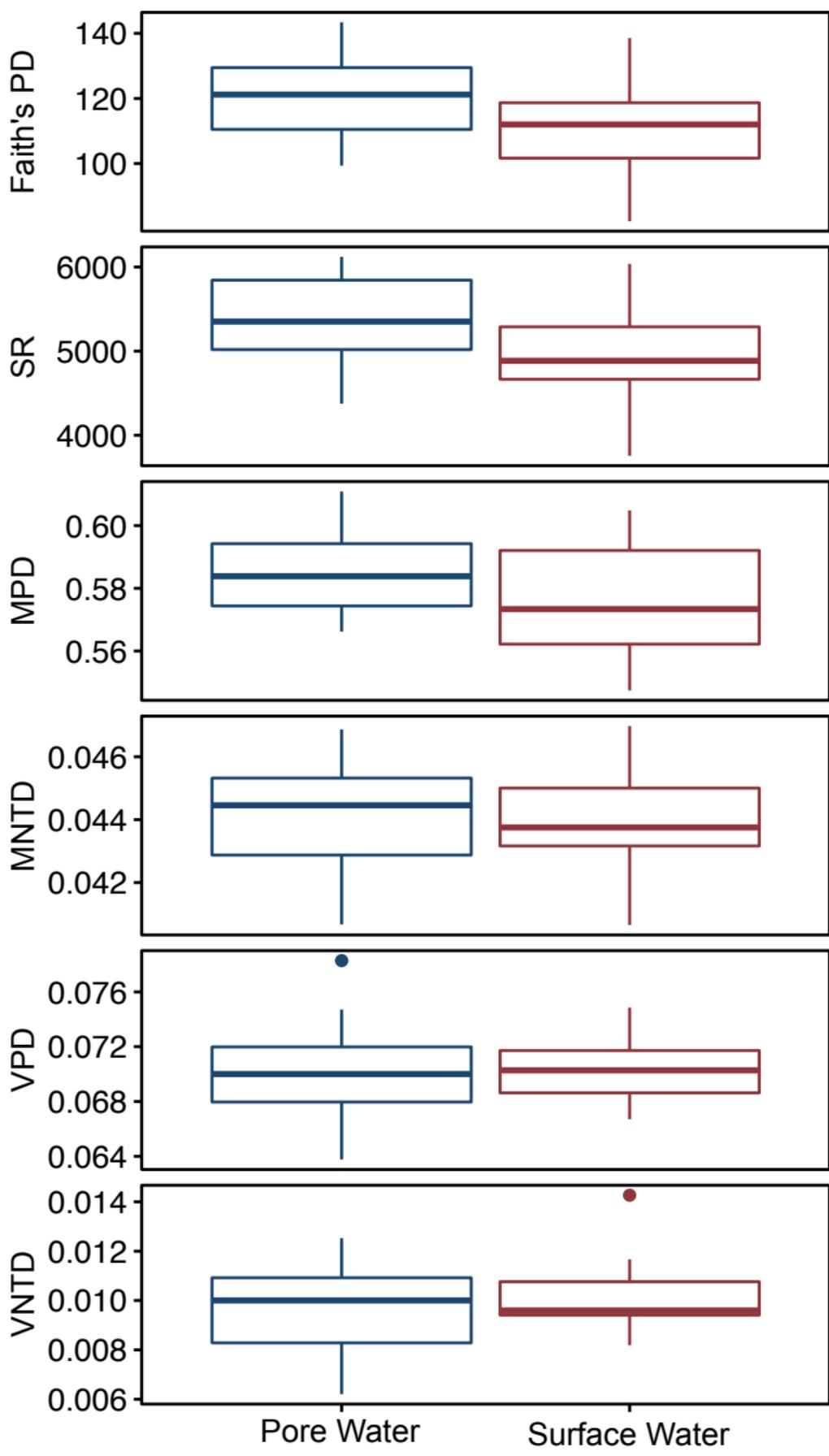
831 using the UPGMA hierarchical clustering method.

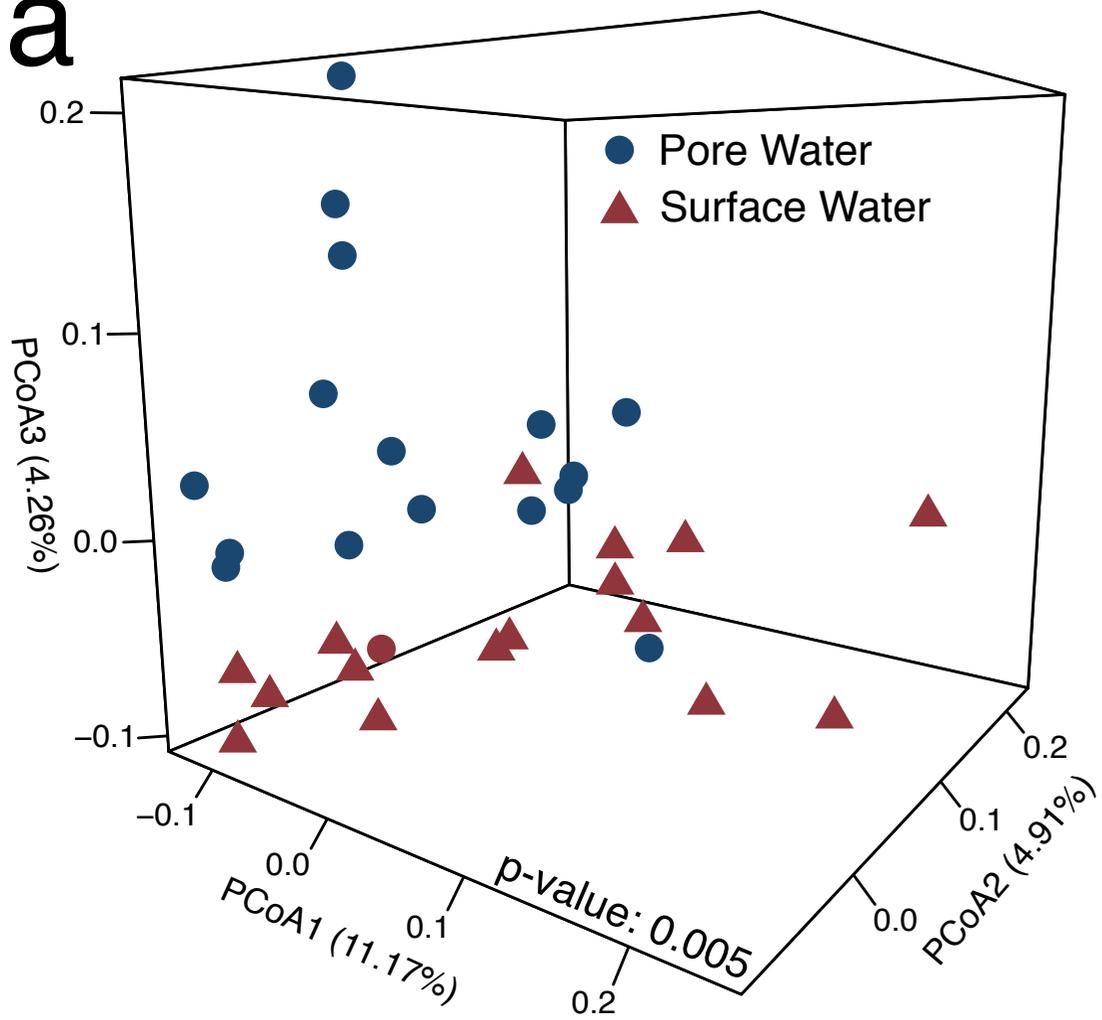
832

833 Supplemental Data 1: Aligned and calibrated FTICR-MS report generated using Formularity.







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