

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

3

4 **Authors:** Robert E. Danczak^{1*}, Amy E. Goldman¹, Rosalie K. Chu², Jason G. Toyoda², Vanessa
5 A. Garayburu-Caruso¹, Nikola Tolić², Emily B. Graham¹, Joseph W. Morad¹, Lupita Renteria¹,
6 Jacqueline R. Wells^{1,3}, Skuyler P. Herzog⁴, Adam S. Ward⁴, James C. Stegen¹

7

8 ¹Pacific Northwest National Laboratory, Washington, USA

9 ²Environmental Molecular Sciences Laboratory, Washington, USA

10 ³Oregon State University, Oregon, USA

11 ⁴O'Neil School of Public Environmental Affairs, Indiana University, Indiana, USA

12

13 * Corresponding Author

14 Email: robert.danczak@pnnl.gov

15

16

17

18

19

20

21

22

23

24

25

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.

41

42

43

44

45

46

47

48

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^{1,3-7}. Recent research has suggested that these ongoing
56 biochemical reactions have a significant influence on river corridor biogeochemistry^{5,6,8}. 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^{4,6}. Moreover, the underlying principles governing the detailed
60 chemistry of DOM are under-investigated⁵. 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^{3,7,9}.

63
64 Recent studies have continued to elucidate principles governing riverine DOM processing^{5,6,10}.
65 Graham *et al.* 2017¹⁰ 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.* 2018⁵ 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.* 2018⁶ 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 chemistry¹¹. 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 implications¹¹. 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.

85

86 Understanding the relative contributions of deterministic and stochastic processes can help reveal
87 mechanisms driving differences in the molecular properties of DOM pools¹¹. Deterministic
88 processes¹² 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 processes¹² 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 metabolites¹²⁻¹⁴.

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 assemblages¹¹. 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^{12,13}. 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^{12,13}. A dominant influence of stochastic processes
110 results in differences in metabolite composition that do not deviate from a random expectation¹⁵.
111 While stochastic processes can also be separated into two classes^{12,13}, 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.*¹¹, 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.* 2012¹⁶, Siefert *et al.* 2013¹⁷, and Dini-Andreote *et al.*
123 2015¹⁴). Using metabolite null modeling, Danczak *et al.*¹¹ 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 research¹⁸⁻²¹. 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 area²² and where riverbed (e.g., hyporheic zone)
138 biogeochemical processes can dominate total respiration²³⁻²⁵. 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^{21,26,27}, 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_{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^{19–21}. 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 β -mean nearest taxon distance (β MNTD) principal
185 coordinate analysis (PCoA) plots showed clear separation between surface and subsurface
186 metabolomes (**Figure 4**; Jaccard p-value – 0.005; β MNTD p-value – 0.02). Furthermore, the

187 Jaccard-based analyses reveal significantly greater differences than did β MNTD. This reflects
188 patterns observed within the dendrograms used in the estimation of β MNTD, but not used to
189 estimate Jaccard; similarities in molecular properties were captured in the dendrogram resulting
190 in decreased separation across the β 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)²⁸⁻³¹. For example,
212 the human gut can have numerous different steady state microbial communities that all exhibit
213 healthy function due to redundant metabolisms³². 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 water⁵. 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.*⁵, 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., NO₃,
248 NO₂, 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 zones⁶. 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 assemblages¹⁵. 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^{13,33}. 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 β -nearest taxon index (β NTI) metric, a

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

272 contributions of these stochastic and deterministic processes^{12,13,15}. 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 genetics³⁴. Recent application of the β 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 likely¹¹.

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)^{13,35}. 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 time¹⁸⁻²¹.

310

311 One of the key biogeochemical differences between surface and subsurface domains in the study
312 system and in other river corridors⁵ 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^{6,36,37}. 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^{6,38,39}. 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 bacteria⁴⁰.

357
358 Similar functional profiles despite divergent taxonomic composition is a common feature in
359 ecological systems^{31,37,41}. Different microbial communities within the human gut or in soil
360 environments will provide similar (if not indistinguishable) contributions to overall ecosystem
361 function³². 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^{6,42}. 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**)^{19,21}. For a detailed description of this
395 study site, please refer to Ward *et al.*²¹ and Wondzell *et al.*¹⁹. Briefly, WS01 is a shallow, low-
396 order, headwater stream which is hydrologically connected to the surrounding terrestrial
397 environment^{19,21}. The river corridor is forested, and evapo-transpiration drives diel fluctuations
398 in stream discharge (**Figure 1**)^{26,27}. 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^{21,26,27}.

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 μm 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 μL injection volume.
431 Standards were made from Spex CertiPrep (Metuchen, NJ, 08840) 1000 mg/L anion standards.
432 NO₂ 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 SO₄ standards were diluted in the range of 0.16 to 80 ppm. NO₃ 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.*⁴³. 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 (–CH₂ 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 Formularity⁴⁴, an in-house software, following the
473 Compound Identification Algorithm^{45–47}. 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 ftmsRanalysis⁴⁸ 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^{49–52}. Samples that were run at both ion accumulation times were
483 combined; given that different IATs will detect different compounds⁵³, 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.*¹¹. First, biochemical
493 transformations were identified within the dataset according to the procedure employed by
494 Breitling *et al.*⁵⁴, Bailey *et al.*⁵⁵, Graham *et al.*^{6,10}, and Garayburu-Caruso *et al.*⁴². 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)⁵⁶. 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**)^{11,57,58}. 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.*¹¹ 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.*¹¹. All metabolites were treated on a
532 presence/absence basis – peak intensities were not used due to charge competition^{47,52}. Richness
533 measurements and Jaccard-based dissimilarity metrics (*vegdist*, ‘vegan’ package 2.5-6)⁵⁶ 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)⁵⁹, 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)^{60–65}. β -mean nearest taxon distance (β MNTD) was measured using the
539 *comdistnt* function in the picante R package⁵⁹. Jaccard dissimilarity and β MNTD results were
540 visualized using a principal coordinates analysis (PCoA; *pcoa*, ‘ape’ package v5.3)⁶⁶.

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 assemblages¹¹. Specifically, the β -Nearest Taxon Index (β NTI) was calculated to measure the
545 influence of stochastic and deterministic assembly processes^{12,13,15}. β NTI was estimated for each
546 pairwise assemblage comparison. To do so, a null distribution of 999 β MNTD values were
547 generated and compared to the observed β MTND 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^{13,14}. 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 βNTI 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)⁵⁶. All correlations were Spearman-based and were performed using the
565 *rcorr* function (‘Hmisc’ package v4.2)⁶⁷. All boxplots and scatter/line plots were generated using
566 the ‘ggplot2’ R package (v3.2.1)⁶⁸; three-dimensional ordinations were generated using the
567 ‘plot3D’ R package (v1.1.1)⁶⁹.

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

575 **References**

- 576 1. Cole, J. J. *et al.* Plumbing the global carbon cycle: Integrating inland waters into the
577 terrestrial carbon budget. *Ecosystems* **10**, 171–184 (2007).
- 578 2. Regnier, P. *et al.* Anthropogenic perturbation of the carbon fluxes from land to ocean. *Nat.*
579 *Geosci.* **6**, 597–607 (2013).
- 580 3. Battin, T. J. *et al.* The boundless carbon cycle. *Nat. Geosci.* **2**, 598–600 (2009).
- 581 4. Wohl, E., Hall, R. O., Lininger, K. B., Sutfin, N. A. & Walters, D. M. Carbon dynamics of
582 river corridors and the effects of human alterations. *Ecol. Monogr.* **87**, 379–409 (2017).
- 583 5. Stegen, J. C. *et al.* Influences of organic carbon speciation on hyporheic corridor
584 biogeochemistry and microbial ecology. *Nat. Commun.* **9**, 585 (2018).
- 585 6. Graham, E. B. *et al.* Multi 'omics comparison reveals metabolome biochemistry, not
586 microbiome composition or gene expression, corresponds to elevated biogeochemical
587 function in the hyporheic zone. *Sci. Total Environ.* **642**, 742–753 (2018).
- 588 7. Zarnetske, J. P., Bouda, M., Abbott, B. W., Saiers, J. & Raymond, P. A. Generality of
589 Hydrologic Transport Limitation of Watershed Organic Carbon Flux Across Ecoregions
590 of the United States. *Geophys. Res. Lett.* **45**, 11,702–11,711 (2018).
- 591 8. Boye, K., Herrmann, A. M., Schaefer, M. V., Tfaily, M. M. & Fendorf, S. Discerning
592 Microbially Mediated Processes During Redox Transitions in Flooded Soils Using Carbon
593 and Energy Balances. *Front. Environ. Sci.* **6**, (2018).
- 594 9. Wohl, E. & Pfeiffer, A. Organic carbon storage in floodplain soils of the U.S. prairies.
595 *River Res. Appl.* **34**, 406–416 (2018).
- 596 10. Graham, E. B. *et al.* Carbon Inputs From Riparian Vegetation Limit Oxidation of
597 Physically Bound Organic Carbon Via Biochemical and Thermodynamic Processes. *J.*

- 598 *Geophys. Res. Biogeosciences* **122**, 3188–3205 (2017).
- 599 11. Danczak, R. E. *et al.* Unification of environmental metabolomics with metacommunity
600 ecology. *Ecol. Lett.*
- 601 12. Stegen, J. C. *et al.* Quantifying community assembly processes and identifying features
602 that impose them. *ISME J.* **7**, 2069–79 (2013).
- 603 13. Stegen, J. C., Lin, X., Fredrickson, J. K. J. K. & Konopka, A. E. Estimating and mapping
604 ecological processes influencing microbial community assembly. *Front. Microbiol.* **6**, 1–
605 15 (2015).
- 606 14. Dini-Andreote, F., Stegen, J. C., van Elsas, J. D. & Salles, J. F. Disentangling mechanisms
607 that mediate the balance between stochastic and deterministic processes in microbial
608 succession. *Proc. Natl. Acad. Sci.* **112**, E1326–E1332 (2015).
- 609 15. Stegen, J. C., Lin, X., Konopka, A. E. & Fredrickson, J. K. Stochastic and deterministic
610 assembly processes in subsurface microbial communities. *ISME J.* **6**, 1653–1664 (2012).
- 611 16. Swenson, N. G. *et al.* Temporal turnover in the composition of tropical tree communities:
612 Functional determinism and phylogenetic stochasticity. *Ecology* **93**, 490–499 (2012).
- 613 17. Siefert, A., Ravenscroft, C., Weiser, M. D. & Swenson, N. G. Functional beta-diversity
614 patterns reveal deterministic community assembly processes in eastern North American
615 trees. *Glob. Ecol. Biogeogr.* **22**, 682–691 (2013).
- 616 18. Kasahara, T. & Wondzell, S. M. Geomorphic controls on hyporheic exchange flow in
617 mountain streams. *Water Resour. Res.* **39**, SBH 3-1-SBH 3-14 (2003).
- 618 19. Wondzell, S. M. Effect of morphology and discharge on hyporheic exchange flows in two
619 small streams in the Cascade Mountains of Oregon, USA. *Hydrol. Process.* **20**, 267–287
620 (2006).

- 621 20. Ward, A. S., Schmadel, N. M., Wondzell, S. M., Gooseff, M. N. & Singha, K. Dynamic
622 hyporheic and riparian flow path geometry through base flow recession in two headwater
623 mountain stream corridors. *Water Resour. Res.* **53**, 3988–4003 (2017).
- 624 21. Ward, A. S., Schmadel, N. M. & Wondzell, S. M. Time-Variable Transit Time
625 Distributions in the Hyporheic Zone of a Headwater Mountain Stream. *Water Resour. Res.*
626 **54**, 2017–2036 (2018).
- 627 22. Buffington, J. M. & Tonina, D. Hyporheic exchange in mountain rivers II: Effects of
628 channel morphology on mechanics, scales, and rates of exchange. *Geogr. Compass* **3**,
629 1038–1062 (2009).
- 630 23. Fellows, C. S., Valett, H. M. & Dahm, C. N. Whole-stream metabolism in two montane
631 streams: Contribution of the hyporheic zone. *Limnol. Oceanogr.* **46**, 523–531 (2001).
- 632 24. Alexander, R. B., Boyer, E. W., Smith, R. A., Schwarz, G. E. & Moore, R. B. The role of
633 headwater streams in downstream water quality. *J. Am. Water Resour. Assoc.* **43**, 41–59
634 (2007).
- 635 25. Argerich, A. *et al.* Comprehensive multiyear carbon budget of a temperate headwater
636 stream. *J. Geophys. Res. Biogeosciences* **121**, 1306–1315 (2016).
- 637 26. Wondzell, S. M., Gooseff, M. N. & McGlynn, B. L. Flow velocity and the hydrologic
638 behavior of streams during baseflow. *Geophys. Res. Lett.* **34**, L24404 (2007).
- 639 27. Wondzell, S. M., Gooseff, M. N. & McGlynn, B. L. An analysis of alternative conceptual
640 models relating hyporheic exchange flow to diel fluctuations in discharge during baseflow
641 recession. *Hydrol. Process.* **24**, 686–694 (2010).
- 642 28. Rosenfeld, J. S. Functional redundancy in ecology and conservation. *Oikos* **98**, 156–162
643 (2002).

- 644 29. Hubbell, S. P. Neutral theory in community ecology and the hypothesis of functional
645 equivalence. *Funct. Ecol.* **19**, 166–172 (2005).
- 646 30. Shipley, B., Vile, D. & Garnier, E. From Plant Traits to Plant Communities: A Statistical
647 Mechanistic Approach to Biodiversity. *Science (80-.)*. **314**, 812–814 (2006).
- 648 31. Louca, S. *et al.* Function and functional redundancy in microbial systems. *Nat. Ecol. Evol.*
649 **2**, 936–943 (2018).
- 650 32. Arumugam, M. *et al.* Enterotypes in the landscape of gut microbial community
651 composition. *Nature* **3**, 1–12 (2013).
- 652 33. Zhou, J. & Ning, D. Stochastic Community Assembly: Does It Matter in Microbial
653 Ecology? *Microbiol. Mol. Biol. Rev.* **81**, e00002-17 (2017).
- 654 34. Hubbell, S. P. *The unified neutral theory of biodiversity and biogeography*. (Princeton
655 University Press, 2001).
- 656 35. Leibold, M. A. *et al.* The metacommunity concept: a framework for multi-scale
657 community ecology. *Ecol. Lett.* **7**, 601–613 (2004).
- 658 36. Graham, E. B. *et al.* Microbes as engines of ecosystem function: When does community
659 structure enhance predictions of ecosystem processes? *Front. Microbiol.* **7**, 1–10 (2016).
- 660 37. Hall, E. K. *et al.* Understanding how microbiomes influence the systems they inhabit. *Nat.*
661 *Microbiol.* **3**, 977–982 (2018).
- 662 38. Frossard, A., Gerull, L., Mutz, M. & Gessner, M. O. Disconnect of microbial structure and
663 function: Enzyme activities and bacterial communities in nascent stream corridors. *ISME*
664 *J.* **6**, 680–691 (2012).
- 665 39. Graham, E. B. & Stegen, J. C. Dispersal-Based Microbial Community Assembly
666 Decreases Biogeochemical Function. *Processes* **5**, 65 (2017).

- 667 40. Gnanaprakasam, E. T. *et al.* Microbial community structure and arsenic biogeochemistry
668 in two arsenic-impacted aquifers in Bangladesh. *MBio* **8**, 1–18 (2017).
- 669 41. Louca, S. *et al.* High taxonomic variability despite stable functional structure across
670 microbial communities. *Nat. Ecol. Evol.* **1**, 1–12 (2017).
- 671 42. Garayburu-Caruso, V. *et al.* Carbon limitation leads to thermodynamic regulation of
672 aerobic metabolism. *bioRxiv* (2020). doi:10.1101/2020.01.15.905331
- 673 43. Dittmar, T., Koch, B., Hertkorn, N. & Kattner, G. A simple and efficient method for the
674 solid-phase extraction of dissolved organic matter (SPE-DOM) from seawater. *Limnol.*
675 *Oceanogr. Methods* **6**, 230–235 (2008).
- 676 44. Tolić, N. *et al.* Formularity: Software for Automated Formula Assignment of Natural and
677 Other Organic Matter from Ultrahigh-Resolution Mass Spectra. *Anal. Chem.* **89**, 12659–
678 12665 (2017).
- 679 45. Kujawinski, E. B. & Behn, M. D. Automated analysis of electrospray ionization fourier
680 transform ion cyclotron resonance mass spectra of natural organic matter. *Anal. Chem.* **78**,
681 4363–4373 (2006).
- 682 46. Minor, E. C., Steinbring, C. J., Longnecker, K. & Kujawinski, E. B. Characterization of
683 dissolved organic matter in Lake Superior and its watershed using ultrahigh resolution
684 mass spectrometry. *Org. Geochem.* **43**, 1–11 (2012).
- 685 47. Tfaily, M. M. *et al.* Sequential extraction protocol for organic matter from soils and
686 sediments using high resolution mass spectrometry. *Anal. Chim. Acta* **972**, 54–61 (2017).
- 687 48. Bramer, L. M. & White, A. ftmsRanalysis: Analysis and visualization tools for FT-MS
688 data. R package version 1.0.0. (2019). Available at: [https://github.com/EMSL-](https://github.com/EMSL-Computing/ftmsRanalysis)
689 [Computing/ftmsRanalysis](https://github.com/EMSL-Computing/ftmsRanalysis).

- 690 49. Hughey, C. A., Hendrickson, C. L., Rodgers, R. P., Marshall, A. G. & Qian, K. Kendrick
691 Mass Defect Spectrum: A Compact Visual Analysis for Ultrahigh-Resolution Broadband
692 Mass Spectra. *Anal. Chem.* **73**, 4676–4681 (2001).
- 693 50. Koch, B. P. & Dittmar, T. From mass to structure: an aromaticity index for high-
694 resolution mass data of natural organic matter. *Rapid Commun. Mass Spectrom.* **20**, 926–
695 932 (2006).
- 696 51. LaRowe, D. E. & Van Cappellen, P. Degradation of natural organic matter: A
697 thermodynamic analysis. *Geochim. Cosmochim. Acta* **75**, 2030–2042 (2011).
- 698 52. Tfaily, M. M. *et al.* Advanced solvent based methods for molecular characterization of
699 soil organic matter by high-resolution mass spectrometry. *Anal. Chem.* **87**, 5206–5215
700 (2015).
- 701 53. Cao, D. *et al.* Ion accumulation time dependent molecular characterization of natural
702 organic matter using electrospray ionization-fourier transform ion cyclotron resonance
703 mass spectrometry. *Anal. Chem.* **88**, 12210–12218 (2016).
- 704 54. Breitling, R., Ritchie, S., Goodenowe, D., Stewart, M. L. & Barrett, M. P. Ab initio
705 prediction of metabolic networks using Fourier transform mass spectrometry data.
706 *Metabolomics* **2**, 155–164 (2006).
- 707 55. Bailey, V. L., Smith, A. P., Tfaily, M., Fansler, S. J. & Bond-Lamberty, B. Differences in
708 soluble organic carbon chemistry in pore waters sampled from different pore size
709 domains. *Soil Biol. Biochem.* **107**, 133–143 (2017).
- 710 56. Oksanen, J. *et al.* vegan: Community Ecology Package. (2019). Available at:
711 <https://cran.r-project.org/package=vegan>.
- 712 57. Moritz, F., Kaling, M., Schnitzler, J. & Schmitt-Kopplin, P. Characterization of poplar

- 713 metabotypes via mass difference enrichment analysis. *Plant. Cell Environ.* **40**, 1057–1073
714 (2017).
- 715 58. Kaling, M. *et al.* Mycorrhiza-Triggered Transcriptomic and Metabolomic Networks
716 Impinge on Herbivore Fitness. *Plant Physiol.* **176**, 2639–2656 (2018).
- 717 59. Kembel, S. W. *et al.* Picante: R tools for integrating phylogenies and ecology.
718 *Bioinformatics* **26**, 1463–1464 (2010).
- 719 60. Faith, D. P. Conservation evaluation and phylogenetic diversity. *Biol. Conserv.* **61**, 1–10
720 (1992).
- 721 61. Clarke, K. R. & Warwick, R. M. Quantifying structural redundancy in ecological
722 communities. *Oecologia* **113**, 278–289 (1998).
- 723 62. Webb, C. O. Exploring the Phylogenetic Structure of Ecological Communities: An
724 Example for Rain Forest Trees. *Am. Nat.* **156**, 145–155 (2000).
- 725 63. Fine, P. V. A. & Kembel, S. W. Phylogenetic community structure and phylogenetic
726 turnover across space and edaphic gradients in western Amazonian tree communities.
727 *Ecography (Cop.)*. **34**, 552–565 (2011).
- 728 64. Tucker, C. M., Shoemaker, L. G., Davies, K. F., Nemergut, D. R. & Melbourne, B. A.
729 Differentiating between niche and neutral assembly in metacommunities using null
730 models of β -diversity. *Oikos* **125**, 778–789 (2016).
- 731 65. Pearse, W. D. *et al.* pez: phylogenetics for the environmental sciences. *Bioinformatics* **31**,
732 2888–2890 (2015).
- 733 66. Paradis, E. & Schliep, K. ape 5.0: an environment for modern phylogenetics and
734 evolutionary analyses in R. *Bioinformatics* **35**, 526–528 (2019).
- 735 67. Harrell, F. E. Hmisc: Harrell Miscellaneous. R package version 4.2-0. (2019). Available

- 736 at: <https://cran.r-project.org/package=Hmisc>.
- 737 68. Wickham, H. *ggplot2: Elegant Graphics for Data Analysis*. (Springer-Verlag New York,
738 2016).
- 739 69. Soetaert, K. *plot3D: Plotting Multi-Dimensional Data*. R package version 1.1.1. (2017).
740 Available at: <https://cran.r-project.org/package=plot3D>.
- 741 70. Stegen, J. C. *et al.* WHONDRS 48 Hour Diel Cycling Study at HJ Andrews Experimental
742 Forest Watershed 1 (WS1). (2019). doi:10.15485/1509695

743

744 **Acknowledgments**

745 Pacific Northwest National Laboratory is operated by Battelle Memorial Institute for the U.S.
746 Department of Energy under Contract No. DE-AC05-76RL01830. This research was supported
747 by the U.S. Department of Energy (DOE), Office of Biological and Environmental Research
748 (BER), as part of BER's Subsurface Biogeochemistry Research Program (SBR). This
749 contribution originates from the SBR Scientific Focus Area (SFA) at the Pacific Northwest
750 National Laboratory (PNNL). A portion of this work was performed at the Environmental
751 Molecular Science Laboratory User Facility.

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.

762

763 **Correspondence**

764 Correspondence can be directed at Robert.Danczak@pnnl.gov

765

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_{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) β 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: β -nearest taxon index (β NTI) calculations across the watershed. a) Boxplots illustrating
798 differences in β NTI results. Mann Whitney U test significance is indicated in the upper right
799 corner. b) Mean β 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 β 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 β NTI and putative

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

819 transformation relative proportions and β NTI, while Sheet 2 are all correlations between

820 transformation groups and β 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.

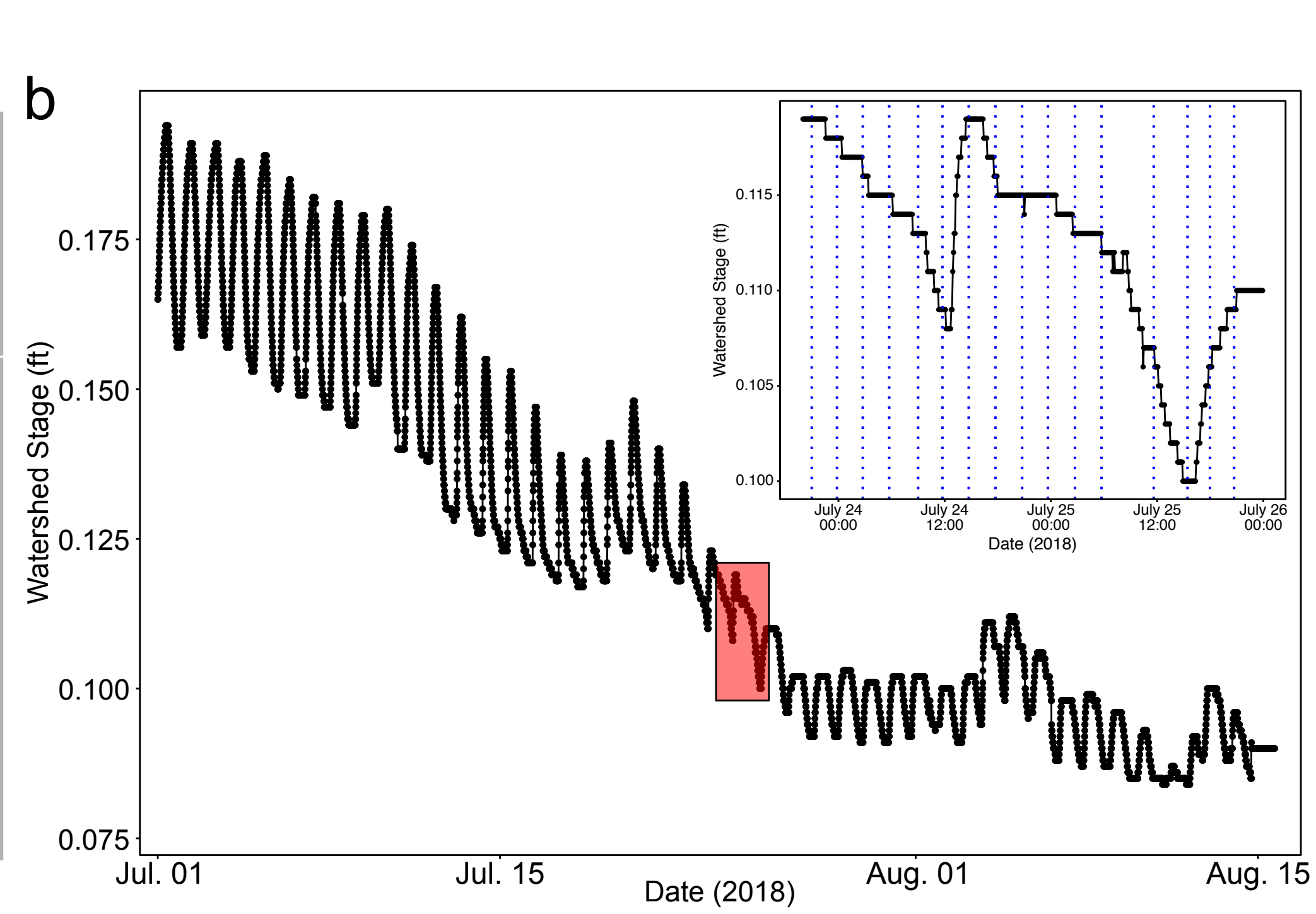
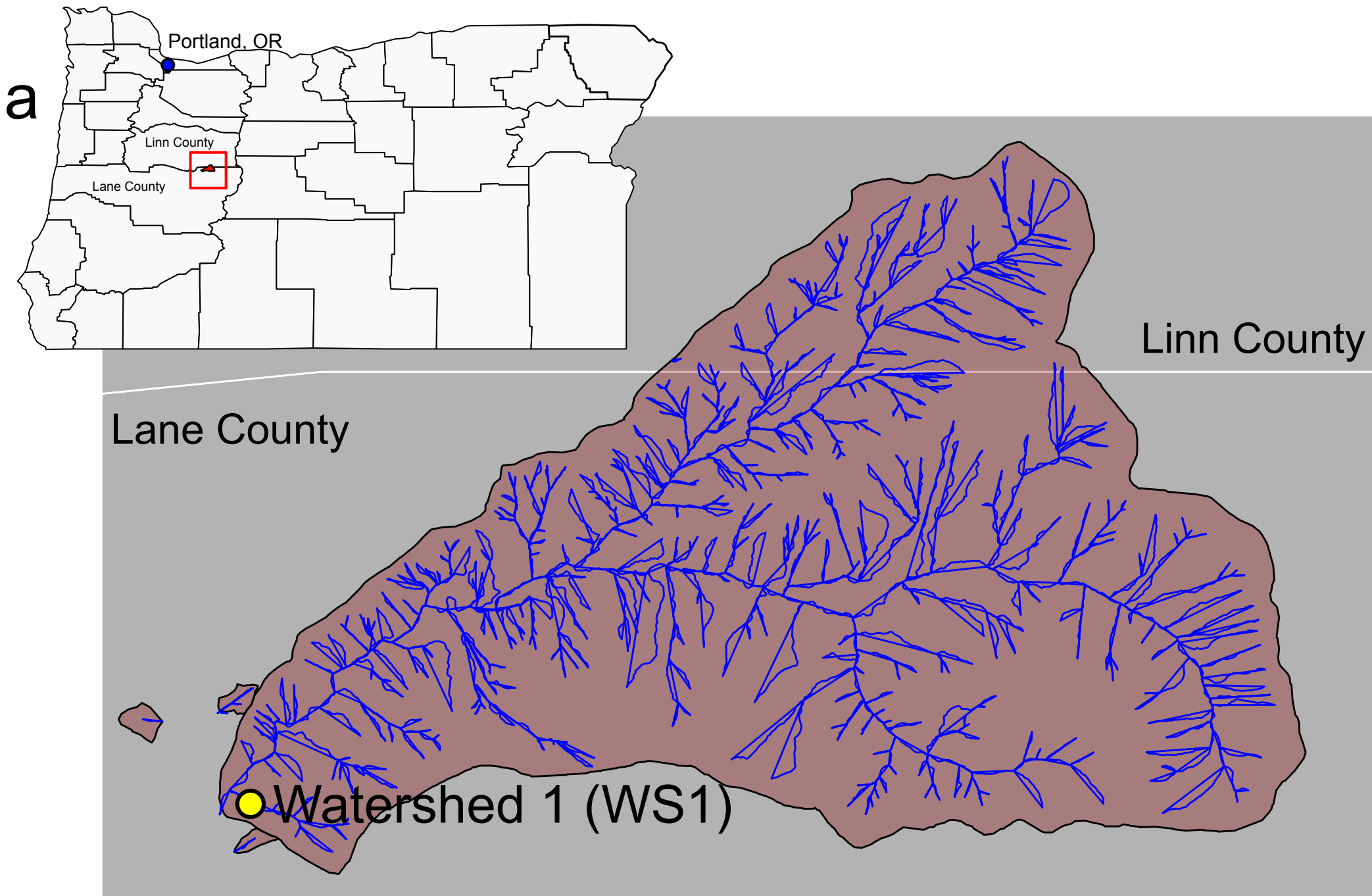
827 This file is used to identify putative biochemical transformations using ultrahigh-resolution mass
828 differences obtained from FTICR-MS datasets.

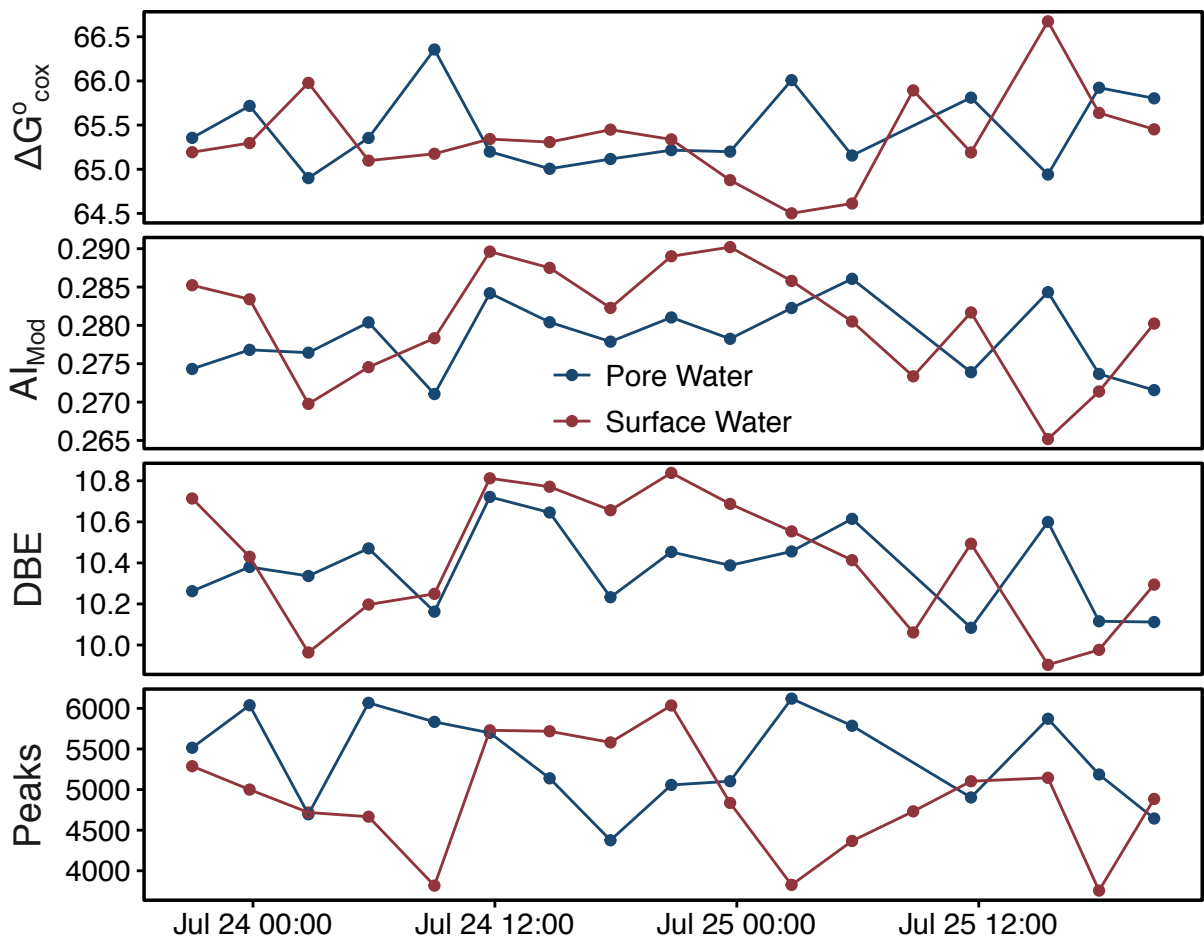
829

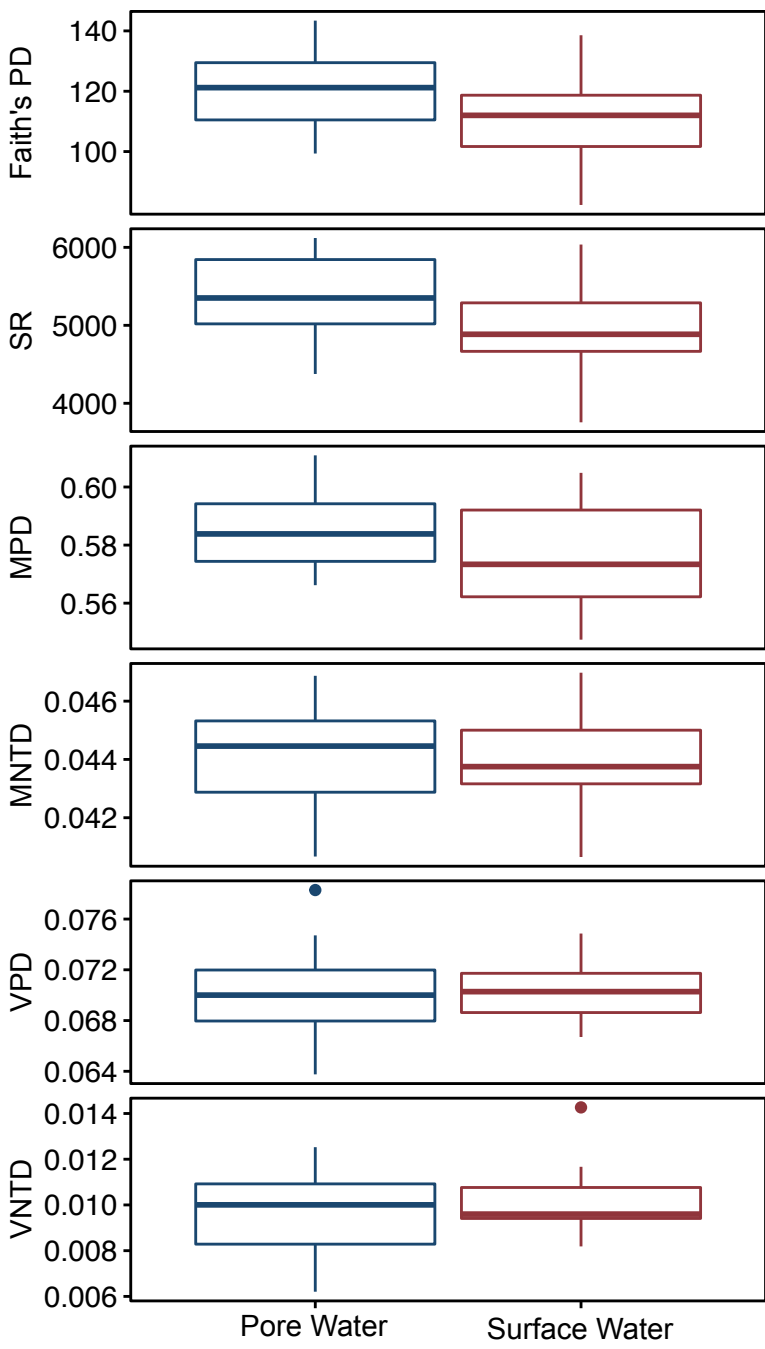
830 Supplemental File 5: The transformation-weighted characteristics dendrogram (TWCD) obtained
831 using the UPGMA hierarchical clustering method.

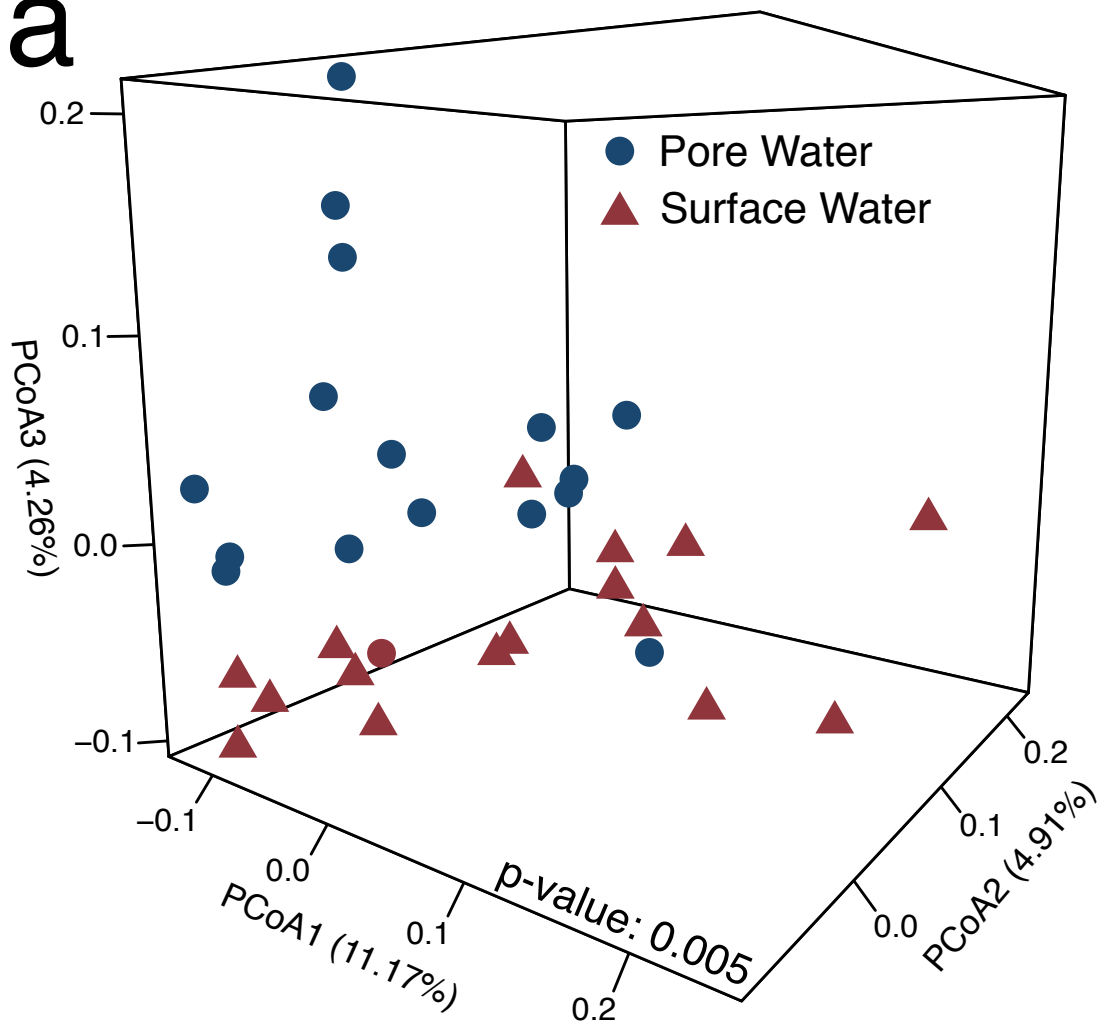
832

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







a**b**