



# 1 Hyporheic Zone Respiration is Jointly Constrained by Organic

## 2 Carbon Concentration and Molecular Richness

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### 8 **Abstract.**

9 River corridors are fundamental components of the Earth system, and their biogeochemistry can be heavily influenced  
10 by processes in subsurface zones immediately below the riverbed, referred to as the hyporheic zone. Within the  
11 hyporheic zone, organic matter (OM) fuels microbial respiration, and OM chemistry heavily influences aerobic and  
12 anaerobic biogeochemical processes. The link between OM chemistry and respiration has been hypothesized to be  
13 mediated by OM molecular diversity, whereby respiration is predicted to decrease with increasing diversity. Here we  
14 test the specific prediction that aerobic respiration rates will decrease with increases in the number of unique organic  
15 molecules (i.e., OM molecular richness, as a measure of diversity). We use publicly available data across the United  
16 States from crowdsourced samples taken by the Worldwide Hydrobiogeochemical Observation Network for Dynamic  
17 River Systems (WHONDORS) consortium. Our continental-scale analyses rejected the hypothesis of a direct limitation  
18 of respiration by OM molecular richness. In turn, we found that organic carbon (OC) concentration imposes a primary  
19 constraint over hyporheic zone respiration, with additional potential influences of OM richness. We specifically  
20 observed respiration rates to decrease nonlinearly with the ratio of OM richness to OC concentration. This relationship  
21 took the form of a constraint space with respiration rates in most systems falling below the constraint boundary. A  
22 similar, but slightly weaker, constraint boundary was observed when relating respiration rate to the inverse of OC  
23 concentration. These results indicate that maximum respiration rates may be governed primarily by OC concentration,  
24 with secondary influences from OM richness. Our results also show that other variables often suppress respiration  
25 rates below the maximum associated with the richness-to-concentration ratio. An important focus of future research  
26 efforts will identify factors that suppress hyporheic zone respiration below the constraint boundaries observed here.

### 27 **1 Introduction**

28 River corridors are key components of the Earth system that connect terrestrial landscapes to the ocean through the  
29 transport and transformation of organic matter (OM) and nutrients (Harvey and Gooseff, 2015; Schlinz and  
30 Schneider, 2000; Schlesinger and Melack, 1981). In addition, river corridors have strong connections to the  
31 atmosphere in terms of significant emissions of greenhouse gasses such as CO<sub>2</sub> (Raymond et al., 2013). Within river  
32 corridors the hyporheic zone (Orghidan, 2010) can have a dominant influence over net metabolism and



33 biogeochemical transformations (Boulton et al., 1998; Naegeli and Uehlinger, 1997; Krause et al., 2011) to a degree  
34 that it can act as the “river’s liver” to remove contaminants (Fischer et al., 2005). Recent work has found that  
35 detailed properties of OM chemistry can significantly influence respiration rates in hyporheic zone sediments  
36 (Stegen et al., 2018; Garayburu-Caruso et al., 2020a; Sengupta et al., 2020; Graham et al., 2018, 2017; Song et al.,  
37 2020a). These observations demonstrate a need to deepen understanding of the relationships between hyporheic  
38 zone biogeochemistry (e.g., respiration rates) and OM chemistry.

39

40 A conceptual hypothesis was recently developed that may provide new insight into the connections between OM  
41 chemistry and biogeochemical rates. More specifically, Lehmann et al. (2020) hypothesize that OM can be protected  
42 from degradation (in part) by high levels of molecular diversity. Biogeochemical rates that depend on OM oxidation  
43 (e.g., aerobic respiration) may therefore be suppressed with increases in the number of unique organic molecules  
44 (referred to here as OM molecular richness). The concept is that high levels of OM molecular richness lead to low  
45 returns-on-investment, relative to the energy invested in building and maintaining the molecular machinery needed  
46 to metabolize any given type of organic molecule. The consequence is low respiration rates. The underlying  
47 mechanism has been proposed to help protect OM in some ecosystems such as deep sea (Arrieta et al., 2015) and  
48 river corridor (Stegen et al., 2018) environments.

49

50 The hypothesis of lower biogeochemical rates with higher OM molecular richness has not been evaluated in  
51 hyporheic zone sediments despite the established connection between OM chemistry and hyporheic zone respiration  
52 rates. We posit that higher levels of hydrologic connectivity in hyporheic zones relative to unsaturated systems (e.g.,  
53 soil) may diminish influences of spatial isolation such as an OM stabilization mechanism (Schmidt et al., 2011),  
54 potentially leading to particularly strong relationships between respiration rates and OM chemistry. In turn, it is  
55 plausible that the hyporheic zone is an ecosystem in which we may find support for the hypothesized negative  
56 relationship between respiration rates and OM molecular richness. Here we test this hypothesis at the continental  
57 scale using publicly available data from the Worldwide Hydrobiogeochemical Observation Network for Dynamic  
58 River Systems (WHONDORS) consortium (Stegen and Goldman, 2018; Garayburu-Caruso et al., 2020b; Toyoda et  
59 al., 2020; Goldman et al., 2020).

## 60 **2 Methods**

### 61 *Sample collection and data generation*

62 During the summer of 2019, the WHONDORS consortium carried out a multi-continent river corridor study to  
63 evaluate interactions between metabolomes, microbial metabolism, biogeochemical function, and ecosystem  
64 features. Garayburu-Caruso et al. 2020b describe details on metadata, sample collection, analysis, and processing of  
65 ultrahigh resolution mass spectrometry data. Briefly, during late July and August 2019 sediment samples were  
66 collected across multiple continents, but the current study focuses on samples collected in the contiguous United  
67 States (ConUS) (Fig. 1). Shallow sediments (~1-5 cm depth) were collected at three separate depositional zones at  
68 each site. The zones were ~ 10 m away from each other and were labeled as upstream, midstream, and downstream.



69 Samples were shipped to the Pacific Northwest National Laboratory (PNNL) campus in Richland, WA (USA) on ice  
70 within 24 hours of collection.

71  
72 In the laboratory, sediments were sieved with a 2 mm sieve, and subsampled into 50 mL conical tubes (Genesee  
73 Scientific Olympus™ Plastics) to separate Field and Incubation aliquots. Note that in the methods provided by  
74 Garayburu-Caruso et al. (2020b) there is an error in the description of the sediment preservation prior to mass  
75 spectrometry analysis. Corrected preservation methods are described immediately below. Sediments from the Field  
76 aliquot were flash frozen in liquid nitrogen immediately after sieving to maintain the sediment characteristics  
77 observed in the field and stored at -80°C until analysis. The Incubation aliquots were not flash frozen immediately;  
78 instead they were kept in the dark inside an environmental chamber at 21°C along with other sediments to be used  
79 for respiration measurements (see below) so that the two sets of sediment samples experienced the same conditions  
80 leading up to the use of the sediment for respiration estimation. The next morning, Incubation aliquots were  
81 retrieved from the environmental chamber, flash frozen in liquid nitrogen, and stored at -80°C until analysis. In our  
82 analyses we used the “Field” sediments to study water-extractable organic carbon concentration and OM chemistry  
83 prior to the respiration incubation. We used the “Incubation” sediments as a check for changes or variation in  
84 organic carbon concentration between Field sediments and those sediments that were actually incubated. As a  
85 quality assurance procedure (detailed below), we removed samples with the largest changes in organic carbon  
86 concentration between Field and Incubation sediments.

87  
88 Field and Incubation sediments were extracted with milli-Q water, and the resulting supernatant from sediment  
89 extractions was filtered through a 0.22 µm sterivex filter (EMD Millipore). Non-purgeable organic carbon (NPOC)  
90 was determined on the supernatant by a Shimadzu combustion carbon analyzer TOC-L CSH/CSN E100V with ASI-  
91 L autosampler. We only included data from sites that had similar NPOC concentrations between the paired Field and  
92 Incubation samples. Our rationale for this approach is based on the assumption that if NPOC is highly variable  
93 across replicate sub-samples (i.e., across paired Field and Incubation samples), the associated sediments used for  
94 respiration measurements may have been highly heterogeneous despite our efforts to homogenize sediments prior to  
95 analyses. In turn, we assume that high heterogeneity may lead to unreliable estimates of NPOC, respiration, and OM  
96 molecular richness for a given site. Focusing analyses on the subset of sites that had relatively good correspondence  
97 in NPOC between Field and Incubation samples is, therefore, a conservative approach aimed at working with only  
98 the most reliable data.

99  
100 To subset the data, we calculated the ratio between Field and Incubation NPOC concentrations within each site. If  
101 the ratio was less than 1, it was inverted so that all ratios were greater than 1. We regressed log-transformed Field  
102 NPOC vs. log-transformed Incubation NPOC, and calculated the  $R^2$  of the associated regression. Log-transformation  
103 was used due to the presence of skewed NPOC distributions. Subsequently, we removed samples in order of their  
104 ratio, starting with the largest ratio (i.e., the largest proportional difference between Field and Incubation NPOC).  
105 Higher  $R^2$  values indicated a tighter relationship between Field and Incubation NPOC, and thus more reliable data.



106 We repeated these steps for all the samples in the Field-Incubation dataset ( $n = 228$ ). We then plotted the  $R^2$  vs. the  
107 number of samples removed and selected a threshold for the number of samples to remove (Fig. S1). The resulting  
108 curve showed that  $R^2$  increased as a function of points removed until it leveled off. This nonlinear saturating  
109 relationship was well-described by a Michaelis-Menten function (Michaelis and Menten, 1913; Johnson and Goody,  
110 2011). In this function, the half saturation constant indicates the resource availability at which half of the maximum  
111 intake is reached (Mulder and Hendriks, 2014). We used the half saturation constant, estimated from fitting the  
112 function to the data in Fig. S1, in a conceptually analogous way. That is, the half saturation constant indicated the  
113 number of samples that would need to be removed to gain half of the maximum potential increase in fit between  
114 Field and Incubation NPOC. This resulted in removing 30 samples, leading to  $R^2 = 0.74$  for the relationship between  
115 Field and Incubation NPOC, which was half way between a minimum  $R^2 = 0.47$  and maximum  $R^2 = 1$ . This  
116 procedure was used to increase the reliability of the OM molecular richness estimates by removing samples that had  
117 the greatest variability in NPOC, which could translate into variability in OM richness as there was a weak but  
118 significant relationship between OM richness and NPOC ( $R^2 = 0.20$ ,  $p < 0.001$ , Fig. S2).

119

#### 120 *Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FTICR-MS)*

121 We used ultrahigh resolution Fourier transform ion cyclotron resonance mass spectrometry (FTICR-MS) to generate  
122 mass spectra of sediment OM pools. Field sediment extracts were normalized to  $1.5 \text{ mg C L}^{-1}$ , acidified to pH 2 and  
123 extracted with solid phase extraction (SPE) PPL cartridges following procedures described by (Dittmar et al., 2008).  
124 Note that all samples were normalized to a consistent NPOC concentration prior to SPE and the same sample  
125 volume was extracted with the same cartridges and resin mass. Since concentrations were normalized prior SPE, we  
126 did not measure extraction efficiency post-extraction. While extraction efficiency will vary across samples, our  
127 approach assumes that variation in extraction efficiency is not systematically linked to respiration rate to such a  
128 degree that the number of detected peaks becomes correlated with respiration. Although we cannot definitively  
129 determine that this assumption is upheld in this dataset, it seems extremely unlikely that the number of observed  
130 peaks would become systematically and spuriously linked to respiration due to variation in extraction efficiency.

131

132 FTICR-MS analyses were carried out at the Environmental Molecular Science Laboratory (EMSL) in Richland, WA  
133 using a 12 Tesla (12T) Bruker Solarix FTICR mass spectrometer (Bruker, Solarix, Billerica, MA, USA) in negative  
134 mode. The method used to assign molecular formulas to FTICR-MS spectra is described in Garayburu-Caruso et al.  
135 (Garayburu-Caruso et al., 2020b). Briefly, Formularity (Tolić et al., 2017) was used to align mass lists generated  
136 using Bruker DataAnalysis V4.2. Resulting reports were processed using ftmsRanalysis (Bramer et al., 2020). It is  
137 important to note that FTICR-MS is a non-targeted approach to reliably identify molecular formulas of organic  
138 molecules with masses, but it is not quantitative and does not provide information about the structure of the  
139 molecular formulas identified. Our analyses on the Field FTICR-MS data only included samples that passed through  
140 the subsetting process described above based on Field and Incubation NPOC. We calculated OM richness as the  
141 total number of unique peaks present in one sample.

142



143

144

145 *Incubations and respiration rates*

146 Respiration rates were determined following methods described by Garayburu-Caruso et al. (2020a). Sieved  
147 sediments were subsampled into 40 mL clear glass vials (I-Chem amber VOA glass vials) with a 0.5 cm diameter  
148 factory calibrated oxygen sensor dot (Fibox 3; PreSens GmbH, Regensburg, Germany). Vials with sediments and  
149 unfiltered water from each site were kept in the dark inside the environmental chamber at a 21°C until next day  
150 incubations. Reactors consisted of 10 mL of sieved sediments and ~30-35 mL of aerated unfiltered water with no  
151 headspace, shaken at 250 rpm for 2 hours. Dissolved oxygen (DO) was measured noninvasively every 15 min for  
152 the first hour and every 30 min during the second hour using an oxygen optical meter (Fibox 3; PreSens GmbH,  
153 Germany) to read the oxygen sensor dots on the vials. Respiration rates were calculated as the slope of the linear  
154 regression between DO concentration and incubation time for each reactor and further normalized per gram of  
155 sediment in each reactor. Normalized and not-normalized rates are reported in this manuscript.

156

157 *Statistical analysis*

158 All statistical analyses were completed using R (version 3.6.3)(R Core Team, 2021) with  $p < 0.05$  as the significance  
159 threshold. We used ordinary least squares regressions (function “lm”) to evaluate relationships between respiration  
160 rates and OM richness or NPOC. While not initially expected, we observed an apparent non-linear constraint-based  
161 relationship between respiration rate and the inverse of NPOC. To evaluate the statistical significance of the  
162 constraint boundary, we subdivided the 1/NPOC data into 10 even bins and found the maximum respiration rate in  
163 each of those bins. We then fit a negative exponential function to the relationship between maximum respiration rate  
164 and 1/NPOC. To evaluate the potential contribution of OM richness, we used the same approach to regress  
165 respiration rate against the ratio of OM richness to NPOC concentration. Base functions in R and ggplot2  
166 (Wickham, 2016) were used for these analyses and associated plotting.

167

168 Scripts necessary to reproduce the primary results of this manuscript are available at

169 [https://github.com/WHONDRS-Hub/Respiration\\_and\\_OM\\_Richness/](https://github.com/WHONDRS-Hub/Respiration_and_OM_Richness/). Goldman et al. (2020) provides the raw,  
170 unprocessed FTICR-MS data and respiration rate data. FTICR-MS data used in this manuscript were processed  
171 following instructions provided in the Goldman et al. (2020) data package.

172

173 **3 Results and Discussion**

174 Both respiration rates and OM molecular richness varied significantly across samples, providing a useful dataset to  
175 study the hypothesized negative relationship between these two variables. More specifically, the distribution of  
176 aerobic respiration rates revealed a broad range of rates that were highly skewed for rates that were either not  
177 normalized (Fig. 2A) or were normalized (Fig. 2B) per gram of sediment. These skewed distributions indicate the  
178 potential for biogeochemical “hot spots” (McClain et al. 2003) or “control points” (Bernhardt et al. 2017) at the  
179 continental scale. The distribution for OM molecular richness (i.e., number of identified organic molecules)



180 appeared to be multimodal, but dominated by one primary peak (Fig. 2C). OM molecular richness ranged from  
181 ~2000-5000 peaks, and we took advantage of this variation to evaluate the relationship between OM richness and  
182 respiration rates.

183

184 We did not observe a clear negative relationship between sediment aerobic respiration rates and OM molecular  
185 richness, which rejected the hypothesis that higher OM richness will suppress respiration (Fig. 3A,C). The data in  
186 Fig.3 suggest there may instead be a peak in maximum respiration rate near intermediate levels of OM molecular  
187 richness. There may, therefore, be an optimal level of OM molecular richness that enables high respiration rates, but  
188 does not guarantee elevated rates, leading to a unimodal constraint space. Regressions based on maximum  
189 respiration rates across the OM richness axis were not significant, however (Fig. 3). These results further reject the  
190 hypothesis of any direct relationship between respiration rate and OM richness.

191

192 While we did not observe a direct link between respiration and OM richness, extending the Lehmann et al. (2020)  
193 hypothesis revealed a potential influence of OM richness, after controlling for water soluble organic carbon (OC)  
194 concentration, measured as NPOC. That is, we posit that any connection between OM richness and respiration is  
195 likely modified by the amount of OC. The magnitude of OM richness relative to the concentration of OC could,  
196 therefore, provide a stronger constraint over respiration than OM richness alone. High ratios indicate high levels of  
197 OM richness relative to the amount of OC, while low ratios indicate low levels of OM richness relative to the  
198 amount of OC. In the context of the Lehmann et al. (2020) hypothesis, respiration would therefore be expected to  
199 decrease with increasing richness-to-concentration ratios.

200

201 Consistent with this extended hypothesis, we find that maximum respiration rates decreased with increasing  
202 richness-to-concentration ratios (Fig. 4). This suggests that at the continental scale OM molecular richness may  
203 indirectly influence aerobic respiration rates. However, the influence of OM richness is likely to be relatively minor.  
204 That is, maximum respiration rate was also well-explained simply to the inverse of OC concentration (Fig. 5). Note  
205 that the relationship between respiration rates and OC concentrations was relatively weak (Fig. S3). The statistical  
206 models using the richness-to-concentration ratio are technically better models as they have higher  $R^2$  values than  
207 models using only the inverse of OC concentration (cf. Figs. 4, 5). We also note that both types of models are  
208 univariate, so there is no penalty for multiple explanatory variables. The bulk of variation in maximum respiration  
209 rates (~90%) is, however, explained simply by the inverse of OC concentration.

210

211 We infer that OC concentration could impose a primary constraint over maximum respiration rates, with OM  
212 richness potentially contributing additional constraints. As such, any influences of OM richness over respiration  
213 rates in hyporheic zone sediments are likely modulated by OC concentration. This is conceptually consistent with  
214 observations in marine (Arrieta et al., 2015) and river corridor systems (Stegen et al., 2018). That is, when OM  
215 molecular richness is high relative to OC concentration, the probability of a microbe repeatedly encountering the  
216 same type of molecule is minimized. In that case, the costs of maintaining metabolic machinery to metabolize any



217 specific type of molecule may outweigh the energy gains (Arrieta et al., 2015). When costs outweigh gains,  
218 respiration is expected to be minimized, which is consistent with the respiration constraint boundary to be lowest at  
219 the highest richness-to-concentration ratios. In addition, the constraint boundary was non-linear, which is most likely  
220 due to the fact that respiration rates cannot be below zero such that increasingly large richness-to-concentration  
221 ratios cannot further suppress respiration.

222  
223 The combined influences of OM richness and OC concentration are realized as a non-linear constraint space with the  
224 vast majority of measured respiration rates falling well below the constraint boundary. This indicates that in most  
225 cases, additional controls over respiration drive respiration below its potential maximum. Discerning these  
226 additional controls is an important avenue of future work. For example, it would be useful to evaluate the degree to  
227 which microbial community diversity, composition, biomass, and/or functional potential are related to deviations  
228 from the constraint boundary. In addition, the FTICR-MS data used here provides presence-absence of organic  
229 molecules, but not relative abundances of organic molecules. Accounting for among-molecule variation in  
230 concentrations could provide insights into factors driving respiration below the constraint boundary. There are many  
231 potential influences spanning physical (e.g., sediment texture), chemical (e.g., mineralogy), and biological (e.g.,  
232 fungal-to-bacterial ratios) that require investigation in context of the constraint discovered here.

233  
234 The outcomes of our study are useful for guiding models towards and away from features and processes that need to  
235 be represented to enable predictions of river corridor biogeochemical function. For large scale models, it appears  
236 there is a constraint envelope for hyporheic zone respiration rates that is related primarily to OC concentration and  
237 even more strongly to OM richness relative to OC concentration. The richness-to-concentration ratio offers a simple  
238 way to represent variation in maximum respiration rates across river corridors. Furthermore, for sites without  
239 estimates of OM richness, our results suggest that variation in maximum respiration rate could be reasonably  
240 estimated via OC concentration alone. Additionally, the constraint spaces observed here could be included in models  
241 more mechanistically whereby they would emerge from the representation of how microbial metabolism is  
242 influenced by both OM molecular richness and OC concentration. The constraint spaces could, alternatively, be  
243 included more phenomenologically in models through probabilistic sampling respiration rates within the constraint  
244 space. There may also be additional aspects of OM molecular richness and chemical diversity more broadly (e.g.,  
245 thermodynamic properties, elemental ratios) that influence respiration and other biogeochemical rates in hyporheic  
246 zone sediments (e.g., Garayburu-Caruso et al., 2020a; Song et al., 2020b). There is a need to examine such  
247 possibilities at both local and global scales.

248  
249 **4 Code availability:** Scripts necessary to reproduce the primary results of this manuscript are available at  
250 [https://github.com/WHONDRS-Hub/Respiration\\_and\\_OM\\_Richness/](https://github.com/WHONDRS-Hub/Respiration_and_OM_Richness/).

251  
252 **5 Data availability:** Data were published previously in Goldman et al. (2020).  
253



254 **6 Author contributions:** JCS and VAG-C conceptualized the study, VAG-C performed analyses with feedback  
255 from JCS, RED processed some of the data, AEG managed the sampling campaign, and LR, JMT, and JW  
256 processed samples. JCS and VAG-C drafted the initial manuscript and all authors contributed to revisions.  
257

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259

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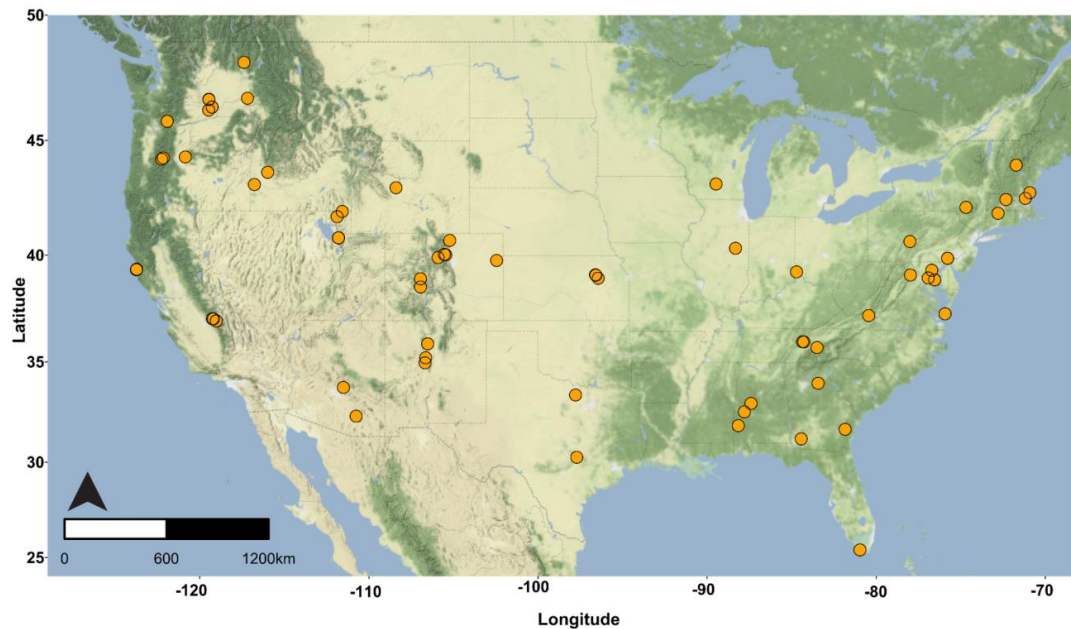
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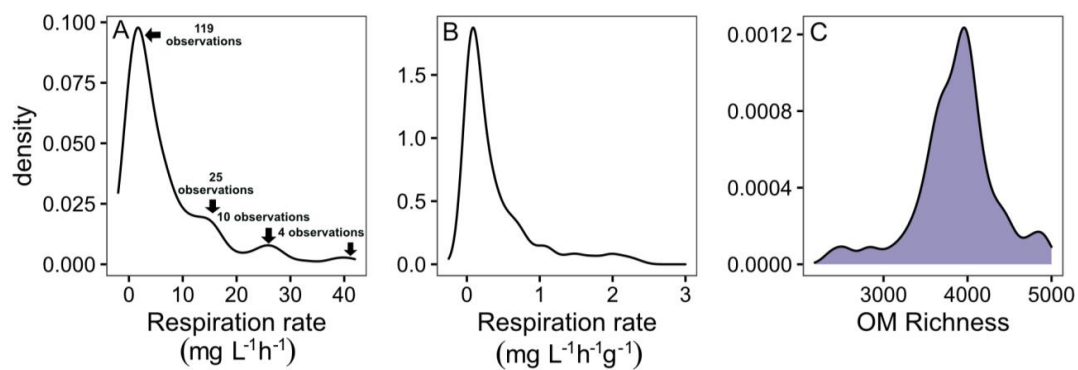
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360 **Figures**  
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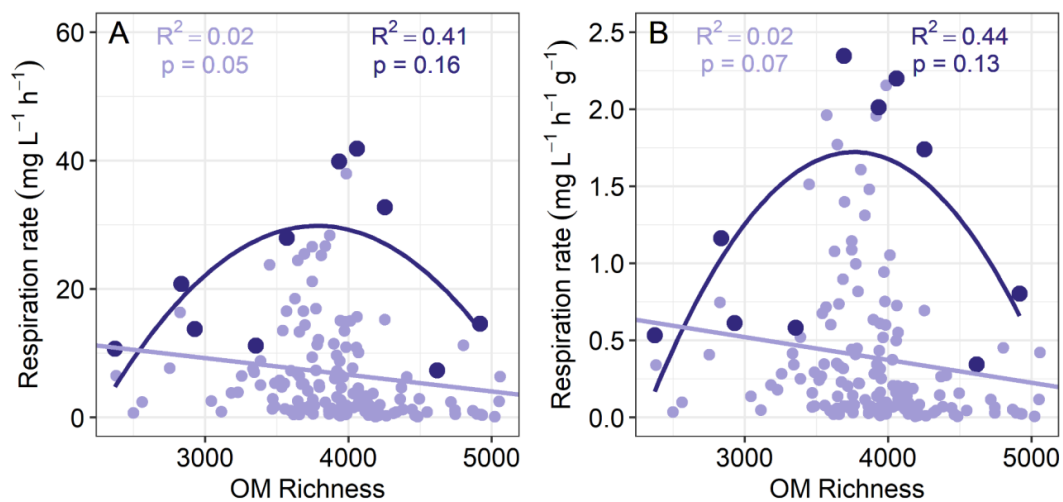


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363 **Figure 1.** Spatial distribution of sampling locations. At each location three sediment samples were collected from locations  
364 distributed along an upstream-downstream gradient within a single stream reach. The map was generated by Sophia McKeever  
365 using QGIS, and the base map is copyrighted: ©OpenStreetMap contributors 2022. The map is distributed under the  
366 Open Data Commons Open Database 504 License (ODbL) v1.0.  
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**Figure 2.** Density plots of aerobic respiration measured as oxygen consumption that was either not normalized relative to sediment mass (A) or normalized by sediment mass (B). Panel (C) is a density plot for the number of unique peaks identified in sediment samples, which we refer to as OM richness.



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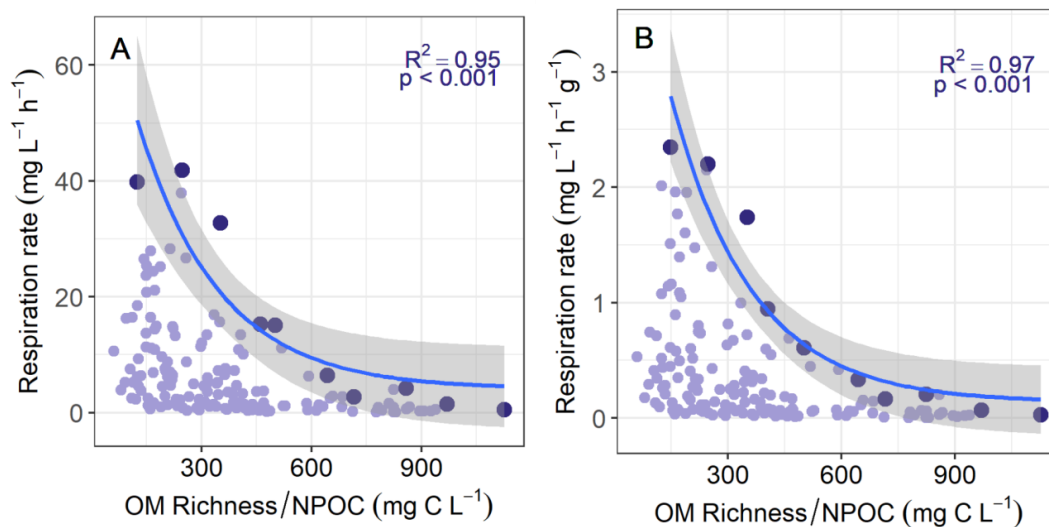
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**Figure 3.** Sediment aerobic respiration as a function of OM richness. Respiration was measured as oxygen consumption and was either not normalized (A) or normalized by sediment mass (B). Quadratic regression models based on maximum respiration rates are shown in dark purple while linear regression models based on all respiration values are shown in light purple. Maximum respiration rates were found by subdividing each horizontal axis into 10 even bins. In all cases the models provided poor fits to the data.



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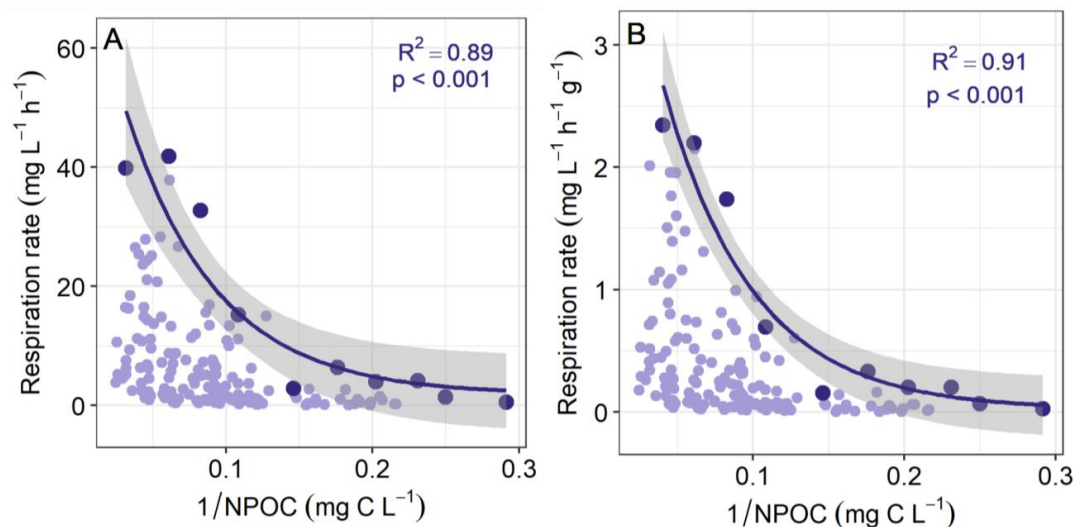
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**Figure 4.** Maximum sediment respiration rate decreased with increasing values of ratio of OM molecular richness to non-purgeable organic carbon concentration (NPOC). Panels A and B are for respiration that was either not normalized or normalized by sediment mass, respectively. Maximum respiration rates (shown in the darker colors) were found by subdividing each horizontal axis into 10 even bins. All other respiration rates and the corresponding richness-to-concentration ratios are shown in lighter colors. Solid lines represent negative exponential models fit to the maximum respiration rates, with shaded areas indicating 95% confidence intervals. Statistics for each model are provided on each panel.



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**Figure 5.** Maximum sediment respiration decreased with the inverse of non-purgeable organic carbon concentration (NPOC). Panels A and B are for respiration that was either not normalized or normalized by sediment mass, respectively. Maximum respiration rates (shown in the darker colors) were found by subdividing each horizontal axis into 10 even bins. All other respiration rates and the corresponding 1/NPOC values are shown in lighter colors. Solid lines represent negative exponential models fit to the maximum respiration rates, with shaded areas indicating 95% confidence intervals. Statistics for each model are provided on each panel.