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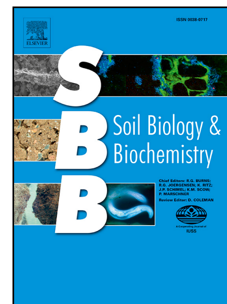

Sources of abiotic hydrolysis of chromogenic substrates in soil enzyme assays: storage, termination, and incubation

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1 **Title**

2 Sources of abiotic hydrolysis of chromogenic substrates in soil enzyme assays: storage,
3 termination, and incubation

4

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10

11 **Keywords**

12 phosphatase; aminopeptidase; cellulase; glucosidase; sulfatase

13

14 **Abstract**

15 Colorimetric assays of enzyme activities using *para*-nitrophenol (*p*NP) and *para*-nitroanilide
16 (*p*NA) substrates are commonly employed in soil science, but these substrates are susceptible
17 to non-enzymatic (i.e., abiotic) hydrolysis. We evaluated abiotic hydrolysis of 10 *p*NP- and *p*NA-
18 linked substrates stored over seven days in two matrices of water and modified universal buffer,
19 and with two alkalization methods of 0.5 M NaOH and 0.1 M Tris. We then compared the
20 magnitude of abiotic versus enzymatic hydrolysis of substrates for two soils with high and low
21 enzyme activity. Finally, we quantified substrate abiotic hydrolysis during the incubation (1-2 h
22 at 37 °C). Abiotic hydrolysis of stored substrate solutions remained relatively constant across 7
23 days, and the base type used in alkalization had a much stronger effect on abiotic hydrolysis
24 than storage time or matrix. Abiotic hydrolysis was generally least for substrates dissolved in
25 water with Tris alkalization and greatest when dissolved in modified universal buffer with NaOH
26 alkalization. The extent of abiotic hydrolysis varied by substrate, and in general was least for
27 ester substrates and greatest for amide substrates. Abiotic hydrolysis was as low as <0.7% for
28 the glycosidic substrate used to assay β-N-acetyl-glucosaminidase, and as high as 52-57% for
29 amide substrates used to assay aminopeptidases. The magnitude of abiotic hydrolysis was
30 more appreciable, and in some cases greater, than total substrate hydrolysis for the soil with
31 overall low enzyme activities. Finally, appreciable abiotic hydrolysis occurred during the
32 incubation, indicating that commonly employed control for non-enzymatic *p*NP or *p*NA products
33 in which substrate solution is added to a soil after the assay incubation is not appropriate. In
34 order to minimize abiotic hydrolysis, we recommend these colorimetric assays of enzyme

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35 activities be terminated with 0.1 M Tris, not 0.5 M NaOH; a secondary but important decrease in
36 abiotic hydrolysis can also be achieved for water-only assays. To accurately control for abiotic
37 hydrolysis in soil enzyme assays, incubated soil-free substrate-only controls should be used.
38
39 **1. Introduction**
40 Soil hydrolytic enzymes catalyze the mineralization of organic carbon (C), nitrogen (N),
41 phosphorus (P), and sulfur (S) (Allison and Vitousek, 2005; Falkowski et al., 2008). The
42 activities of soil enzymes are often used as indicators of soil nutrient cycling in unmanaged
43 ecosystems and agroecosystems (1994), and serve to link soil microbial communities with
44 nutrient pools (Sinsabaugh et al., 2005). Soil enzyme activities are generally assayed using two
45 major types of artificial substrates: chromogenic and fluorometric (Deng et al., 2017).
46 Chromogenic substrates, namely *para*-nitrophenol (*p*NP) and *para*-nitroanilide (*p*NA) substrates,
47 yield spectrophotometrically quantifiable products that enable high throughput assays of soil
48 enzyme activities (Deng et al., 2017). Despite recent evaluations of soil enzyme methodology,
49 several methodological questions on these artificial substrates persist (Nannipieri et al., 2018).
50 In particular, abiotic hydrolysis – non-enzymatic degradation – of substrates is an understudied
51 but known artifact in chromogenic assays (Margenot et al., 2018). Chromogenic substrates can
52 be categorized by the major type of bond hydrolyzed by the target enzyme: ester, glycosidic and
53 amide bonds (Fig. 1). Given strong differences in the thermodynamic stability of these bonds
54 and thus susceptibility to non-enzymatic hydrolysis (e.g., nucleophilic attack), the type of bond
55 may influence abiotic hydrolysis of chromogenic substrates by alkalization thought to 'terminate'
56 assays. If unaccounted for, abiotic hydrolysis can result in overestimation of enzyme activity
57 because the product generated from the substrate will be falsely attributed to enzymatic activity
58 (Fig. 2).
59
60 One source of abiotic hydrolysis of substrates is time in storage of substrate solutions. There
61 are gaps in knowledge regarding shelf-life of solutions of *p*NP- and *p*NA-linked substrates
62 (German et al., 2011). For fluorometric assays that also employ artificial substrates based on an
63 aromatic scaffold, however, significant effects of storage have been identified. For example, 4-
64 methylumbelliferyl (MUF)-linked substrate solution reportedly degraded after 3 days in cold (4
65 °C) storage (DeForest, 2009). As a result, for MUF-linked substrates, it is recommended that
66 solutions be made within 24 hours of assays, and that substrate solutions not be stored for more
67 than three days (DeForest, 2009). For chromogenic enzyme assays employing *p*NP- and *p*NA-

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68 linked substrates, however, it is not known how long substrate solutions can be stored, nor how
69 this may vary by substrate.
70
71 Abiotic hydrolysis of chromogenic substrates may also be induced by the method of alkalization
72 used to terminate the assay. Early evaluations of base types used in alkalization suggested that
73 NaOH could induce abiotic hydrolysis of *p*NP-linked substrates used to assay β-glucosidase
74 (Tabatabai, 1994), phosphodiesterase (Browman and Tabatabai, 1978), and lipase (Margesin et
75 al., 2002) relative to tris(hydroxymethyl)aminomethane (Tris). Relative to 0.1 M Tris, 0.5 M
76 NaOH increased abiotic hydrolysis of pNP-linked substrates used to assay sulfatase (Klose et
77 al., 2011), leading to recommendations that the weak base be used instead of NaOH for these
78 enzymes. This suggests that the wide diversity of *p*NP- and *p*NA-linked chromogenic substrates
79 may be differentially susceptible to such hydrolysis.
80
81 The potential effects of matrix type on substrate abiotic hydrolysis remain unclear, though
82 compared to water the high ionic strength of buffers is likely to influence substrate stability in
83 solution (Bisswanger, 2014). Buffers, commonly modified universal buffer (MUB) in
84 chromogenic enzyme assays but sometimes acetate or Tris buffers (Sinsabaugh and Linkins,
85 1990; Acosta-Martínez and Tabatabai, 2000), are proposed to control solution pH during the 1-2
86 h incubation period of the assay. However, the enzyme-specific and thus substrate-specific pH
87 of buffer is based on an (assumed) optimal pH for enzyme activity (Tabatabai, 1994) that may
88 not be universally applicable to all soils (Wade et al., in review). Given evidence that the pH
89 optimum is specific to each soil (Margesin et al., 2002; Turner, 2010) and using buffers to
90 enforce an assumed universal pH optimum can lead to measuring inaccurate activities (Wade et
91 al., in review), the use of water has been proposed as an alternative to buffer (Yavitt et al.,
92 2004; Chaer et al., 2009; Lessard et al., 2013). Water-based assays of soil enzymes are also
93 thought to better reflect in situ soil pH (Burns et al., 2013) and avoid buffer-induced artifacts
94 such as inhibition or stimulation of enzyme activity (Kiss et al., 1975; Holford, 1979). Since the
95 assay matrix (e.g., buffer or water) can influence the effect of base type used for alkalization
96 with soil and/or substrates, it is likely that abiotic hydrolysis under NaOH and Tris are also
97 influenced by the assay matrix.
98
99 A final factor that could potentially influence abiotic hydrolysis of chromogenic substrates is the
100 incubation of the assay. Chromogenic soil enzymes assays typically entail incubation of the soil
101 and substrate solution for 1-2 h at 37 °C (Tabatabai and Bremner, 1969; Margenot et al., 2018).

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102 However, despite early demonstrations of the need to conduct incubated soil-free controls of
103 substrate abiotic hydrolysis (Jackson, 2013), widely used protocols (e.g., Tabatabai, 1994) do
104 not account for abiotic hydrolysis during incubation, because substrate is added at the end of
105 the incubation to a soil-only incubated control. Though this approach accounts for abiotic
106 hydrolysis of substrate prior to the incubation and post-incubation (i.e., alkalization), it does not
107 account for abiotic hydrolysis that could occur during the incubation period of the assay. Limited
108 protocols have described this for the chromogenic β-glucosidase substrate at a relatively low
109 assay temperature of 20 °C (Sinsabaugh and Linkins, 1987), though the amount of abiotic
110 hydrolysis was not reported. The relatively high temperature of 37 °C used for many soil
111 enzyme assays (Tabatabai, 1994) could risk abiotic hydrolysis of chromogenic substrates, as
112 many substrates are temperature sensitive and require storage at lower temperatures ranging
113 from -20 to 4 °C.
114
115 This study quantified potential sources of abiotic hydrolysis of chromogenic substrates used to
116 assay soil enzymes. A selection of substrates was used to quantify abiotic hydrolysis under
117 common practices for storage, matrix, assay incubation and base type used in alkalization. First,
118 we monitored abiotic hydrolysis of 10 *p*NP- and *p*NA-linked substrates over seven days using
119 two matrices and two bases used in alkalization. We expected that the substrates would differ in
120 the magnitude of abiotic hydrolysis during storage, and that the extent of abiotic hydrolysis
121 would increase with storage time. Second, we determined the magnitude of abiotic hydrolysis in
122 *p*NP- and *p*NA-linked substrates. We hypothesized that alkalization using NaOH would increase
123 abiotic hydrolysis relative to Tris, and that the proportion of substrate abiotically hydrolyzed
124 would be substrate-specific and largely predicated on major bond types (glycosidic, ester, and
125 amide). We further hypothesized the magnitude of abiotic hydrolysis to be more appreciable
126 relative to enzyme activity for soils with lower activities compared to soils with higher enzyme
127 activities. Lastly, to evaluate potential degradation of substrates during the assay, we compared
128 abiotic hydrolysis of substrates in solution that were incubated (37°C for 1-2 h) before
129 alkalization versus immediate alkalization. We expected that incubating *p*NP- and *p*NA-linked
130 substrates would increase abiotic hydrolysis, and that this would also be substrate-specific.
131

132 **2. Methods**

133 *2.1. Sites and soil sampling*

134 Two soils with relatively low and high organic matter content were used to furnish low and high
135 enzyme activities, which generally scale with soil organic carbon (Sinsabaugh et al., 2008). The

136 two soils were the Flanagan series (soil$_{High}$; fine, smectitic, mesic Aquic Argiudolls; 40° 4' 57.30''
137 N, 88° 13' 29.22'' W) and Cisne series (soil$_{Low}$; fine, smectitic, mesic Mollic Albaqualfs; 38° 5'
138 45.22'' N, 88° 50' 40.98'' W), both under maize (*Zea mays*) agriculture in Illinois, USA (Table 1).
139 Previous assessments of activities of hydrolytic enzymes in these soils identified large
140 differences in activities, enabling their use to furnish examples of how the relative magnitude of
141 abiotic hydrolysis of substrates may be of varying significance depending on enzyme activity.
142 The surface depth of plowed A horizons were sampled at both sites. Soils were sampled as a
143 composite at each site, at 0-5 cm depth using a soil knife in a 0.2 ha plot (n=3) for soil$_{High}$ and at
144 0-10 cm depth using an auger in a 1.0 ha plot (n=16) for soil$_{Low}$. Historical mean annual
145 precipitation at the location of soil$_{High}$ is 1045 mm and mean annual temperature is 10.9 °C.
146 Historical mean annual precipitation at the location of soil$_{Low}$ is 1100 mm and mean annual
147 temperature is 13.3 °C. Soils were air-dried (25 °C) and sieved to < 2 mm. Though air-drying of
148 soil can change the absolute value of measured enzyme activities (Bandick and Dick, 1999),
149 relative differences in enzyme activities among soils are still preserved (Wallenius et al., 2010)
150 and thus provide contextualization of abiotic hydrolysis for the purpose of this study.
151
152 *2.2. Enzyme substrates*
153 A total of eight *p*NP- and two *p*NA-linked substrates were evaluated (Table 2), corresponding to
154 enzymes that are generally interpreted as C-cycling (n=4), C-/N-cycling (n=1), N-cycling (n=2),
155 P-cycling (n=2) and S-cycling (n=1). The *p*NP moiety is linked via a glycosidic bond in the C-
156 cycling and C-/N-cycling enzyme substrates, via a phosphoester bond in the P-cycling enzyme
157 substrates, and via a sulfate ester bond in the S-cycling enzyme substrates. The *p*NA moiety is
158 linked via an amide bond in the N-cycling enzyme substrates. Abbreviations, defined in Table 2,
159 refer to the substrate evaluated.
160
161 *2.3. Shelf study methods*
162 Substrate solutions were prepared in either MUB or deionized water and stored at 4°C for seven
163 days. Substrate concentrations were determined based on recommended or commonly reported
164 practices (Table 2). A stock solution of MUB was created as described by (Turner, 2010) and
165 stored for no more than 2 weeks at 4°C. Substrate solutions were subjected to alkaline
166 'termination' (i.e., alkalization) on days 0, 1, 4, and 7. For each substrate, at each timepoint
167 mean abiotic hydrolysis was calculated using replicated stored solutions (n=4; Table S1a). To
168 homogenize the solution and redissolve potential substrate and/or hydrolyzed product
169 precipitates, substrate solutions were stirred vigorously and pipetted from the resulting

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170 suspension. Similar to the abiotic hydrolysis blanks, 4 mL of MUB or water (18.2 mΩ cm^{-1}) were
171 added to 50 mL centrifuge tubes, followed by 1 mL of substrate solution. Samples were
172 immediately alkalized without incubation using either 4 mL of 0.5 M NaOH or 0.1 M Tris (pH 12),
173 as well as 1 mL of 0.5 M $CaCl_2$. Samples were then centrifuged for 105 sec at 17,968 g and 0.2
174 mL of supernatant was used for colorimetric determination of *p*NP or *p*NA (410 nm) in 96-well
175 plates (0.360 mL well volume) by spectrophotometry (Biotek Instruments Inc.). We used *p*NA
176 calibrations with absorbance at 410 nm, which is on the shoulder of the maximum absorbance
177 at 380 nm but avoids interference from unhydrolyzed substrate <360 nm (Kato et al., 1978;
178 Lottenberg and Jackson, 1983; Perez de Castro et al., 1988).
179
180 *2.4. Soil enzyme assay methods*
181 Substrate solutions were prepared on the same day of the assay (< 6 hours) using either MUB
182 or water (18.2 mΩ cm^{-1}). The general procedure to assay enzyme activities was based on
183 Tabatabai and Bremner (1969) with modifications by Tabatabai (1994) and Margenot et al.
184 (2018). Approximately 1.00 ± 0.02 g of air-dried soil in a 50 mL centrifuge tube was combined
185 with either 4 mL of MUB or deionized water, followed by 1 mL of substrate solution using the
186 same matrix in quadruplicates. Mixtures were swirled for 10 sec and incubated at 37°C for 1 or
187 2 h, depending on prescribed assay durations (Table 2). Alkalization was then administered by
188 adding either 4 mL of 0.5 M NaOH (Tabatabai, 1994) or 0.1 M Tris (pH 12) (Klose et al., 2003;
189 Klose et al., 2011), as well as 1 mL of 0.5 M $CaCl_2$. Centrifugation and colorimetry were
190 performed as described above. Abiotic hydrolysis was estimated using soil-free controls, in
191 which the same total volume and substrate concentration as soil assays were incubated for the
192 same duration at 37°C.
193
194 For evaluating abiotic hydrolysis during the soil enzyme assays, average abiotic hydrolysis for
195 treatments (2×2 factorial of matrix × alkalization base) was calculated using n=8 replicates for
196 BG, GAL, PDE, NAG and SUL, and n=4 replicates for PME, LAP, GAP, MAN, and CBH (Table
197 S1b). Differences in replicates were due to performing evaluations twice for one set of five
198 substrates. Total hydrolysis of each substrate, which includes enzymatic and abiotic hydrolysis,
199 was corrected for (1) potential soil-specific artifacts of absorbance from dissolved organic matter
200 using a soil-only control subjected to the same incubation conditions and alkalization treatments
201 (Margenot et al., 2018) and (2) incomplete recovery of product from the soil, performed using
202 single point sorption of 1 mM g^{-1} of *p*NP or *p*NA ((Cervelli et al., 1973; Margenot et al., 2018).
203 After converting the absorbance value to concentration (mM), each sample was corrected for

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204 dilution, dissolved organic matter (DOM), then soil sorption. Each sample was then converted to
205 μmol *p*NP/*p*NA g^{-1} h^{-1} (Formula S1a).
206
207 *2.5 Statistical Analysis*
208 To evaluate the effects of matrix and alkalization base type on the magnitude of abiotic
209 hydrolysis, we performed Kruskal-Wallis rank sum test followed by Dunn's test of multiple
210 comparisons using the *dunnTest()* function in *FSA* package (Ogle et al., 2020) and the *cldList()*
211 function in *rcompanion* package (Mangiafico, 2020) in R software version 4.0.0 in Rstudio
212 version 1.1.463 (RStudio Team, 2016; R Core Team, 2020). To additionally test potential
213 interactions between the effects of matrix and alkalization base type, we performed
214 nonparametric factorial analysis with the aligned rank transform technique using the *ARTool*
215 package in R (Wobbrock et al., 2011; Martin et al., 2020; Kay et al., 2021). Nonparametric tests
216 were conducted due to the severe non-normality and heteroscedasticity of the data even after
217 various transformations. The tests were performed separately for each substrate to compare
218 four treatment combinations (2 matrix type × 2 alkalization base type) for abiotic hydrolysis
219 during the soil enzyme assays. For the substrate solution storage study, the tests were similarly
220 conducted for each substrate at each timepoint.
221
222 To test the hypothesis that abiotic hydrolysis can occur during incubation, we calculated the
223 difference in abiotic hydrolysis between soil-free controls during soil assays and day 0 results
224 from the shelf study. Values were grouped and averaged based on matrix and alkalization base
225 types. To evaluate these differences, pairwise comparisons were conducted using a Wilcoxon-
226 Mann-Whitney test (exact p-value) with NPAR1WAY using SAS v9.4, because assumptions of
227 normality and equality of variances were not met for the majority of observations (SAS Institute
228 Inc., 2013).
229
230 **3. Results**
231 *3.1. Storage of substrate solutions and interactions with matrix and base choice*
232 Substrate degradation over the 7-day storage period depended on the matrix and the type of
233 base used to alkalize substrate solutions, and this effect was greater than storage duration
234 (Table 3; see Table S2 for absolute values). Glycosidic bond substrates expressed lowest
235 abiotic hydrolysis over time under Tris alkalization and ranged 0.01-0.70%. Abiotic hydrolysis
236 was over 5-fold higher with NaOH for GAL, GB, and MAN. For NAG and CBH, abiotic hydrolysis
237 was 1.5- and 2.5-fold higher, respectively, under NaOH alkalization. Changes in abiotic

238 hydrolysis across time were not consistent among the glycosidic substrates. Abiotic hydrolysis
239 of CBH and MAN decreased across 7 days under Tris alkalization. Abiotic hydrolysis of CBH in
240 water over 7 days was greater by 22% when alkalization used NaOH, and by nearly 50% in
241 buffer. In contrast, abiotic hydrolysis increased by 12-14% for NAG across 7 days with Tris
242 alkalization, and decreased by 50% in buffer with NaOH alkalization. Degradation of GAL
243 decreased over 7 days using all methods except in water with Tris alkalization, which increased
244 by 1.5%. Hydrolysis of NAG was unaffected by storage time.
245
246 Ester bond substrates exhibited greater degradation in buffer matrix with NaOH alkalization.
247 Overall, each substrate expressed < 0.9% abiotic hydrolysis throughout 7 days. For PME in
248 buffer, abiotic hydrolysis over the 7 days increased nearly 5-fold when alkalized with NaOH
249 compared to Tris. However, abiotic hydrolysis of PME in water was 28% lower with alkalization
250 by NaOH than Tris. PDE followed a similar trend, as abiotic hydrolysis increased nearly 1.6-fold
251 in buffer under NaOH alkalization, but was an order of magnitude lower in water with NaOH
252 alkalization. Although abiotic hydrolysis of SUL in buffer using NaOH alkalization was highest, it
253 decreased by 7% over the 7 days of storage. When stored in water, SUL degradation under
254 NaOH alkalization was 37% higher than with Tris.
255
256 Amide bond substrates presented the largest magnitudes of abiotic hydrolysis. GAP and LAP
257 expressed maximum abiotic hydrolysis of 35% and 43%, respectively. Similar to the trend of the
258 glycosidic substrates, alkalization with NaOH resulted in higher abiotic hydrolysis. Changes in
259 abiotic hydrolysis across the 7 days were similar for GAP and LAP in water under Tris
260 alkalization, with 14% and 12% increases, respectively. In buffer under Tris alkalization, abiotic
261 hydrolysis increased by 6% from timepoints 0-7 for both GAP and LAP. Degradation of GAP
262 and LAP in buffer with NaOH decreased by 7% and 8%, respectively. However, trends across
263 the 7 days differed between GAP and LAP in water under NaOH alkalization; abiotic hydrolysis
264 increased by 41% for GAP but decreased by 13% for LAP.
265
266 *3.2. Abiotic hydrolysis (% degradation) during soil assays*
267 Glycosidic substrates expressed greatest abiotic hydrolysis when dissolved in buffer and
268 alkalized with NaOH, followed by water with NaOH, buffer with Tris, and water with Tris in
269 descending order across all five substrates (Fig. 3a-b; Table S3). However, the magnitude of
270 abiotic hydrolysis relative to the total hydrolysis in the two soils varied by substrate. For NAG in
271 buffer with NaOH alkalization, abiotic hydrolysis was 22% and 55% of the total hydrolysis in

272 soil$_{High}$ and soil$_{Low}$, respectively. Abiotic hydrolysis was also relatively low for BG in buffer,
273 amounting to 33% in soil$_{High}$ and 73% in soil$_{Low}$ with NaOH alkalization. For these same
274 methods, abiotic hydrolysis of CBH and GAL was greater than CBH total hydrolysis (+11%) and
275 GAL total hydrolysis (+13%) in soil$_{Low}$. Total hydrolysis of MAN was lower than abiotic hydrolysis
276 in both soil$_{High}$ (-4%) and soil$_{Low}$ (-32%).
277
278 Ester substrates also exhibited the highest substrate degradation in buffer with NaOH
279 alkalization (Fig. 3c). However, the relative magnitude of abiotic hydrolysis compared with total
280 hydrolysis similarly varied by substrate. For PDE in buffer with NaOH alkalization, the
281 proportions of total and abiotic hydrolysis in both soils were nearly equivalent. However, abiotic
282 hydrolysis was at most 44% of total hydrolysis of PDE under all other methods. For PME, only
283 6% or less of total hydrolysis was attributable to abiotic hydrolysis across all methods in soil$_{High}$.
284 In soil$_{Low}$, abiotic hydrolysis was at most 11% of total hydrolysis. Using water as the matrix, SUL
285 abiotic hydrolysis was 23% or less of total hydrolysis in both soils. However, abiotic hydrolysis
286 surpassed total hydrolysis assayed with buffer in soil$_{Low}$ with alkalization using Tris by 2% and
287 NaOH by 19%.
288
289 Amide substrates were greatly abiotically hydrolyzed by NaOH alkalization compared to
290 glycosidic or ester substrates, and their associated aminopeptidases most often displayed lower
291 total hydrolysis in comparison to abiotic hydrolysis alone (Fig. 3d). As a result, the magnitude of
292 abiotic hydrolysis of aminopeptidase substrates was comparable or greater than total hydrolysis.
293 Abiotic hydrolysis in both GAP and LAP was up to 99% higher with NaOH relative to Tris
294 alkalization, and all total hydrolysis was over 90% higher with NaOH alkalization. Abiotic
295 hydrolysis of LAP was 58-178% greater than total hydrolysis when assayed with NaOH
296 alkalization. For GAP, abiotic hydrolysis was 22% higher than the total hydrolysis in soil$_{Low}$ in
297 water with NaOH alkalization, and 32% higher than total hydrolysis in soil$_{High}$ using buffer. With
298 Tris alkalization, GAP and LAP abiotic hydrolysis was nearly always lower than 77% of the total
299 hydrolysis in both soils. However, in soil$_{Low}$, LAP hydrolysis was 81% higher than total hydrolysis
300 assayed in water with Tris alkalization.
301
302 *3.3. Abiotic hydrolysis during incubation*
303 Incubation at 37°C generally entailed degradation of the 10 substrates, with greater abiotic
304 hydrolysis of ester bond substrates than glycosidic or amide bond substrates (Table S4). The
305 effect of base type in this comparison represents the abiotic hydrolysis from incubation

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306 combined with the influence of alkalization. Glycosidic bond substrates expressed lower abiotic
307 hydrolysis when incubated in water and subjected to Tris alkalization. However, BG and GAL
308 exhibited lower abiotic hydrolysis when incubated in buffer under Tris alkalization. The largest
309 relative difference with higher abiotic hydrolysis from incubation occurred for MAN incubated in
310 buffer and alkalized with NaOH, with up to 230% more abiotic hydrolysis compared to
311 incubation in water with Tris alkalization. NAG and CBH incubated in buffer with Tris alkalization
312 were the second-most sensitive to the incubation, exhibiting a maximum increase in abiotic
313 hydrolysis of 95% and 99% during incubation, respectively. Under NaOH alkalization, BG and
314 GAL in buffer exhibited up to 59% and 56% more abiotic hydrolysis during incubation than in
315 water.
316
317 Ester bond substrates generally exhibited more abiotic hydrolysis when incubated. Incubation
318 induced abiotic hydrolysis by up to +1,119% for PME and +525% for PDE. In contrast, SUL had
319 the least incubation effect, where relative differences were no more than 49%. Differences
320 between incubated and unincubated substrates for PME and PDE were largest in buffer under
321 NaOH alkalization. For SUL, differences in abiotic hydrolysis due to incubation were largest in
322 buffer under Tris alkalization.
323
324 Differences in degradation between incubated and unincubated samples varied across amide
325 bond substrates. For both LAP and GAP, the magnitudes of differences in hydrolysis (μmol *p*NA
326 g^{-1} h^{-1}) between incubated and unincubated samples were consistently larger when alkalized
327 with NaOH relative to Tris. Although incubation increased GAP abiotic hydrolysis by no more
328 than 12% of the total substrate, this corresponded to a relative increase in abiotic hydrolysis of
329 1% to 96%. For LAP, absolute differences in abiotic hydrolysis during the assay incubation
330 ranged from -7% to 9% depending on matrix and alkalization base, whereas relative differences
331 were between -25% to 22%.
332

333 4. Discussion

334 *4.1. Effect of storage duration on substrate solutions*

335 Abiotic hydrolysis of substrates stored as solutions in water or buffer varied across 7 days, but
336 the choice of base for alkalization had a much larger effect on abiotic hydrolysis than storage
337 duration. Despite the dissimilarities in how much substrate degraded during the 7-day storage
338 period between amide bond substrates compared to the glycosidic and ester bond substrates,
339 nearly all substrates expressed significantly higher abiotic hydrolysis when alkalized with NaOH

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340 (see Section 4.2). Abiotic hydrolysis of substrates with NaOH alkalization was also generally
341 greatest across 7 days when glycosidic and ester substrates were dissolved in buffer compared
342 to water, except MAN and SUL. Our findings suggest that NaOH not only increases abiotic
343 hydrolysis in *p*NP- and *p*NA-linked substrates, but may also increase abiotic hydrolysis of
344 substrates stored for longer time periods and especially when dissolved in buffer.
345
346 Unexpectedly, there were several instances for each substrate bond type in which degradation
347 of the substrate appeared to decrease over time. A true decrease in substrate hydrolyzed over
348 time is not possible since the cleaved bond (Fig. 1) cannot re-form spontaneously. Cases of
349 decreasing abiotic hydrolysis were most common and of higher magnitudes between timepoints
350 0 and 1, and with NaOH alkalization. This was observed in spite of efforts to minimize substrate
351 changes in solution due to settling out or precipitation, by re-suspending the substrate solution
352 prior to measurements taken at each timepoint. Additionally, it is unlikely that this resulted from
353 substrate precipitation because the trends are inconsistent across both base types at a given
354 timepoint. For example, abiotic hydrolysis of GAL in buffer decreased by nearly 0.2% across 7
355 days under NaOH alkalization, but remained consistent under Tris alkalization. If precipitation
356 were substrate-induced, similar trends in abiotic hydrolysis would be expected across both base
357 types. Thus, differences in base type and matrix interactions are likely driving apparent variation
358 in abiotic hydrolysis over storage time. Since estimates of abiotic hydrolysis during storage
359 measured with Tris alkalization in either matrix type were lower than with NaOH alkalization,
360 these rates represent the maximum levels of abiotic hydrolysis during storage time.
361
362
363 *4.2. Effect of alkalization base and matrix choice on abiotic hydrolysis*
364 Aggravation of abiotic hydrolysis by NaOH alkalization overshadowed the effect of storage time,
365 and in soil assays the greater magnitude of abiotic hydrolysis with this strong base led – for
366 some substrates – to large overestimates of soil enzyme activity. For all 10 substrates in this
367 study, alkalization using NaOH yielded higher proportions of abiotic hydrolysis relative to Tris.
368 This may be explained by the strong nucleophilic nature of the hydroxide ion, which via
369 nucleophilic substitution facilitates β-elimination reaction of *p*NP or *p*NA as the leaving group
370 (Goddard and Reymond, 2004; Reymond, 2004). Abiotic hydrolysis was generally highest when
371 the substrate was dissolved in buffer. Thus, observed abiotic hydrolysis does not appear to
372 solely be a result of alkaline pH.
373

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374 Notably, the magnitude of overestimation due to abiotic hydrolysis was strongly substrate-
375 specific. Amide bond substrates expressed the highest proportions of abiotic hydrolysis, which
376 was always greater than total hydrolysis when NaOH was used for alkalization. This implies that
377 accurate enzyme assays using *p*NA-linked substrates may not be feasible with NaOH
378 alkalizations, which could explain why base was not used to terminate aminopeptidase assays
379 in the first applications of this substrate type, which used alanine *para*-nitroanilide (Brown, 1985;
380 López Tomás et al., 2006). The first application of *p*NA-linked substrates in food and medical
381 sciences did not alkalize assays before colorimetry, as this does not appear to be necessary for
382 *p*NA color development (Alvarado et al., 1992; López Tomás et al., 2006). Thus, avoiding
383 alkalization altogether is feasible based on previous studies, and preferable for these amide
384 bond substrates based on the present study.
385
386 Secondary to base effect, matrix choice had an effect on abiotic hydrolysis for several
387 substrates, and this generally differed by substrate bond type. For the (phospho)ester bond
388 substrates (PME, PDE) in the soil assays, a water matrix resulted in higher abiotic hydrolysis
389 compared to buffer + Tris alkalization. However, buffer with Tris alkalization yielded higher
390 abiotic hydrolysis than water with Tris in sulfate ester bond substrate (SUL). All glycosidic bond
391 substrates except GAL underwent greater abiotic hydrolysis when dissolved in buffer for either
392 choice of base for alkalization. Relative to base choice, matrix type does not appear to be a
393 major driver of abiotic hydrolysis to the extent that it compromises measurement of enzymatic
394 hydrolysis.
395
396 *4.3. Interactions of abiotic hydrolysis and soil*
397 For most substrates, we found that the magnitude of abiotic hydrolysis was more appreciable
398 relative to total hydrolysis for $soil_{Low}$. For glycosidic and ester bond substrates, the magnitude of
399 abiotic hydrolysis was proportionally more significant relative to total hydrolysis in $soil_{Low}$ than in
400 $soil_{High}$, and especially so for total hydrolysis measured using PDE, CBH, MAN, and GAL
401 substrates and in buffer with NaOH alkalization. The use of NaOH for alkalization can therefore
402 compromise accuracy of enzyme activity measurements if abiotic hydrolysis corrections are not
403 implemented. To ensure the subtraction of abiotic hydrolysis, soil-free blanks using only
404 substrate should be used in the assay, including incubation. However, because the activity
405 equivalents of abiotic hydrolysis are subtracted directly from enzyme activities, these corrections
406 can produce negative enzymatic activities when abiotic hydrolysis exceeds enzymatic
407 hydrolysis. For both CBH and MAN in a water matrix, it is notable that abiotic hydrolysis alone is

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408 negative when alkalized with Tris. These negative values may be explained by insignificant
409 noise in the absorbance readings, and ultimately represent zero abiotic hydrolysis. Due to the
410 large proportional magnitude of abiotic hydrolysis using NaOH in many cases, Tris alkalization
411 appears to mitigate this risk for most substrates, consistent with previous reports (Browman and
412 Tabatabai, 1978; Margesin et al., 2002) and its more recent proposed use (Klose et al., 2003;
413 Klose et al., 2011).
414
415 Abiotic hydrolysis of both amide substrates was substantially higher than total hydrolysis under
416 NaOH alkalization, though was less appreciable for $soil_{Low}$. Under Tris alkalization, however, the
417 magnitude of abiotic hydrolysis was more appreciable for total hydrolysis in $soil_{Low}$, and it was
418 higher than LAP total hydrolysis in buffer. These instances of abiotic hydrolysis outstripping
419 enzyme activity indicate that it may be not be possible to fully correct for abiotic hydrolysis in low
420 activity soils. This overestimation of abiotic hydrolysis could also be occurring in high activity
421 soils, but is not detectable when the corrected activity estimations do not yield negative enzyme
422 activities.
423
424 Since abiotic hydrolysis measured for substrate solutions without soil present were greater than
425 hydrolysis uncorrected for abiotic sources in soils (i.e., enzymatic + abiotic), our results suggest
426 that abiotic hydrolysis is higher without soil than with soil. In theory, abiotic hydrolysis should be
427 at most equivalent to total hydrolysis (i.e., no enzyme activity in the soil). However, greater
428 abiotic hydrolysis than total hydrolysis in soil assays measured for several substrates indicates
429 that interactions with the soil matrix can protect *p*NP- and *p*NA-linked substrates from abiotic
430 hydrolysis (Stemmer, 2004). In addition, components of the soil matrix, in particular acidic
431 functional groups and exchangeable H^+, could consume the base such as the hydroxide driving
432 abiotic hydrolysis. It does not appear possible to correct for this disparity because measuring
433 soil-based abiotic hydrolysis of artificial substrates without the contribution of enzymatic
434 hydrolysis is challenging given the persistent activity of extracellular enzymes.
435
436 *4.4. Effect of incubation on abiotic hydrolysis*
437 Our comparison of incubated and unincubated substrates indicates that incubation facilitates
438 more abiotic hydrolysis in some substrates. Therefore, the current widespread practice of
439 adding the substrate solution to a soil-only assay *after* incubation (Tabatabai, 1994) does not
440 fully account for all sources of non-enzymatic hydrolysis. Notably, fluorogenic substrates are
441 incubated to account for abiotic hydrolysis during the assay itself (Dick et al., 2018). The 10

442 chromogenic substrates evaluated here generally exhibited higher abiotic hydrolysis than
443 unincubated substrates, with some exceptions depending on matrix and base choice. Though
444 the differences in abiotic hydrolysis were minor (<2%) for all substrates except the amide bond
445 substrate, compared to alkalization effects this is an overlooked source of abiotic hydrolysis that
446 requires accounting for. Our results are consistent with a previous finding that the glycosidic
447 bond substrate of BG did not undergo detectable abiotic hydrolysis during incubation when
448 alkalized with Tris (Eivazi and Tabatabai, 1988). However, this appears to have been assumed
449 to be true for other *p*NP-linked enzymes, and even for BG an earlier study demonstrated minor
450 but detectable abiotic hydrolysis (Hayano, 1973). Though less commonly used to assay
451 aminopeptidases compared to fluorometric assays (e.g., Jian et al., 2016), GAP and LAP
452 expressed considerable abiotic hydrolysis during incubation when alkalized with NaOH,
453 signifying that the elevated temperature of the assay further aggravates the high abiotic
454 hydrolysis observed for this amide bond substrates with NaOH alkalization. Colorimetric assays
455 incubated at temperatures lower than 37˚C (Sinsabaugh and Linkins, 1987; Selmants and Hart,
456 2010) would likely result in lower abiotic hydrolysis of the amide bond substrates. For any
457 temperature, substrate solution incubated without soil should be used to account for abiotic
458 hydrolysis that occurs during this period of the assay.
459
460
461 *4.5. Methodological recommendations to minimize abiotic hydrolysis of pNP/pNA-linked*
462 *substrates*
463 This study demonstrates that several components in the methodology of soil enzyme assays
464 can have substantial effects on abiotic hydrolysis of chromogenic substrates. Though this
465 depends on the enzyme type, and the magnitude of this artifact of enzyme activity
466 overestimation is relatively small compared to enzymatic hydrolysis, general best practices are
467 possible to mitigate abiotic hydrolysis:
468 1. *Abiotic hydrolysis corrections:* Current control methods for abiotic hydrolysis combine
469 correction for dissolved organic matter and abiotic hydrolysis prior to incubation, but
470 overlook abiotic hydrolysis during the incubation. We recommend accounting for abiotic
471 hydrolysis in enzyme activity measurements using incubated, soil-free blanks. This
472 accounts for all sources of non-enzymatic substrate hydrolysis prior to alkalization
473 (Formula S1b).
474 2. *Substrate solution storage:* PME, PDE, SUL, MAN, CBH, BG, GAL, and NAG substrate
475 solutions can be stored at least 7 days prior to the soil enzyme assay in either water or

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476 modified universal buffer. GAP and LAP substrate solutions can be stored at least 7
477 days only if alkalized with 0.1 M Tris.
478 3. *Matrix and alkalization:* We recommend alkalization with 0.1 M Tris (pH 12). Though
479 generally matrix type does not appear to be as major a driver of abiotic hydrolysis, using
480 water also decreases abiotic hydrolysis. Thus, given complex considerations of whether
481 to use water or buffer (Burns et al. 2013) and the assumption of pH optima needed to
482 use buffers (Turner, 2010), the choice to use water as a matrix offers ancillary benefits to
483 mitigating abiotic hydrolysis. However, SUL and GAL may also be used in water and
484 alkalized with 0.5 M NaOH with minimal abiotic hydrolysis. For PDE, CBH, and BG,
485 assays have least abiotic hydrolysis risk when dissolved in water with either alkalization
486 method, or in modified universal buffer with Tris alkalization. Finally, PME and NAG
487 assays may be administered under any of the four combinations of matrix type and
488 alkalization base, including NaOH alkalization in modified universal buffer.
489
490 **5. Conclusion**
491 Abiotic hydrolysis of enzyme substrates based on *p*NP and *p*NA chromophores can significantly
492 decrease the accuracy of soil enzyme activity estimations. The matrix and base used for
493 alkalization have a large effect on abiotic hydrolysis, which can be appreciable or even exceed
494 enzymatic hydrolysis. In order to minimize the magnitude of abiotic hydrolysis, we evaluate
495 hypothesized major sources of abiotic hydrolysis in chromogenic substrate assays. Amide bond
496 substrates are highly sensitive to abiotic hydrolysis, whereas ester and glycosidic bond
497 substrates are least sensitive. In general, abiotic hydrolysis was least when substrates were
498 dissolved in water, instead of MUB, and with alkalization using 0.1 M Tris instead of 0.5 M
499 NaOH. Solutions of the ten substrates evaluated can be stored for up to 7 days, as abiotic
500 hydrolysis during storage appears to be relatively low compared to the method of alkalization.
501 To ensure the accuracy of soil enzyme activity measurements by fully accounting for abiotic
502 hydrolysis, a soil-free, substrate-only control should be subjected to incubation. The
503 recommendations from this study stand to improve comparability of enzyme activities
504 determined across enzyme types and soils.
505
506 **References**
507 Acosta-Martínez, V., Tabatabai, A.M., 2000. Enzyme activities in a limed agricultural soil.
508 Biology and Fertility of Soils 31, 85-91.

509 Allison, S.D., 2008. Allison Lab Protocol: Colorimetric Enzyme Assays. UC Irvine.

Journal Pre-proof

Journal Pre-proof

510 Allison, S.D., Jastrow, J.D., 2006. Activities of extracellular enzymes in physically isolated
511 fractions of restored grassland soils. Soil Biology and Biochemistry 38, 3245-3256.

512 Allison, S.D., Vitousek, P.M., 2005. Responses of extracellular enzymes to simple and complex
513 nutrient inputs. Soil Biology and Biochemistry 37, 937-944.

514 Alvarado, R., Rodriguez-Yunta, M.A., Hoz, L., Garcia de Fernando, G.D., Ordonez, J.A., 1992.
515 Rapid p-Nitroaniline Test for Assessing Microbial Quality of Refrigerated Meat. Journal of Food
516 Science 57, 1330-1331.

517 Bailey, V.L., Fansler, S.J., Smith, J.L., Bolton, H., 2011. Reconciling apparent variability in
518 effects of biochar amendment on soil enzyme activities by assay optimization. Soil Biology and
519 Biochemistry 43, 296-301.

520 Bandick, A.K., Dick, R.P., 1999. Field management effects on soil enzyme activities. Soil
521 Biology and Biochemistry 31, 1471-1479.

522 Bisswanger, H., 2014. Enzyme assays. Perspectives in Science 1, 41-55.

523 Browman, M.G., Tabatabai, M.A., 1978. Phosphodiesterase Activity of Soils1. Soil Science
524 Society of America Journal 42, 284-290.

525 Brown, K.A., 1985. Acid deposition: Effects of sulphuric acid at pH 3 on chemical and
526 biochemical properties of bracken litter. Soil Biology and Biochemistry 17, 31-38.

527 Burns, R.G., DeForest, J.L., Marxsen, J., Sinsabaugh, R.L., Stromberger, M.E., Wallenstein,
528 M.D., Weintraub, M.N., Zoppini, A., 2013. Soil enzymes in a changing environment: Current
529 knowledge and future directions. Soil Biology and Biochemistry 58, 216-234.

530 Cervelli, S., Nannipieri, P., Ceccanti, B., Sequi, P., 1973. Michaelis constant of soil acid
531 phosphatase. Soil Biology and Biochemistry 5, 841-845.

532 Chaer, G.M., Myrold, D.D., Bottomley, P.J., 2009. A soil quality index based on the equilibrium
533 between soil organic matter and biochemical properties of undisturbed coniferous forest soils of
534 the Pacific Northwest. Soil biology and biochemistry 41, 822-830.

535 DeForest, J.L., 2009. The influence of time, storage temperature, and substrate age on potential
536 soil enzyme activity in acidic forest soils using MUB-linked substrates and l-DOPA. Soil Biology
537 and Biochemistry 41, 1180-1186.

538 Deng, S., Dick, R., Freeman, C., Kandeler, E., Weintraub, M.N., 2017. Comparison and
539 standardization of soil enzyme assay for meaningful data interpretation. Journal of
540 Microbiological Methods 133, 32-34.

Journal Pre-proof

541 Dick, R., 1994. Soil Enzyme Activities as Indicators of Soil Quality, Defining Soil Quality for a
542 Sustainable Environment, pp. 107-124.

543 Eivazi, F., Tabatabai, M.A., 1988. Glucosidases and galactosidases in soils. Soil Biology and
544 Biochemistry 20, 601-606.

545 Falkowski, P.G., Fenchel, T., Delong, E.F., 2008. The Microbial Engines That Drive Earth's
546 Biogeochemical Cycles. Science 320, 1034-1039.

547 German, D.P., Weintraub, M.N., Grandy, A.S., Lauber, C.L., Rinkes, Z.L., Allison, S.D., 2011.
548 Optimization of hydrolytic and oxidative enzyme methods for ecosystem studies. Soil Biology
549 and Biochemistry 43, 1387-1397.

550 Goddard, J.-P., Reymond, J.-L., 2004. Recent advances in enzyme assays. Trends in
551 Biotechnology 22, 363-370.

552 Hagmann, D.F., Goodey, N.M., Mathieu, C., Evans, J., Aronson, M.F.J., Gallagher, F., Krumins,
553 J.A., 2015. Effect of metal contamination on microbial enzymatic activity in soil. Soil Biology and
554 Biochemistry 91, 291-297.

555 Holford, I., 1979. Evaluation of soil phosphate buffering indices. Soil Research 17, 495-504.

556 Jackson, C.R., Tyler, H.L., Millar, J.J., 2013. Determination of microbial extracellular enzyme
557 activity in waters, soils, and sediments using high throughput microplate assays. Journal of
558 Visualized Experiments 80, 1-9. doi:10.3791/50399.

559 Jian, S., Li, J., Chen, J., Wang, G., Mayes, M.A., Dzantor, K.E., Hui, D., Luo, Y., 2016. Soil
560 extracellular enzyme activities, soil carbon and nitrogen storage under nitrogen fertilization: A
561 meta-analysis. Soil Biology and Biochemistry 101, 32-43.

562 Kato, T., Nagatsu, T., Kimura, T., Sakakibara, S., 1978. Studies on substrate specificity of X-
563 prolyl dipeptidyl-aminopeptidase using new chromogenic substrates, X-Y-p-nitroanilides.
564 Experientia 34, 319-320.

565 Kay, M., Elkin, L., Higgins, J., Wobbrock, J.O., 2021. ARTool: Aligned Rank Transform for
566 Nonparametric Factorial ANOVAs. R package version 0.11.0.

567 Kiss, S., Drăgan-Bularda, M., Rădulescu, D., 1975. Biological significance of enzymes
568 accumulated in soil, Advances in agronomy. Elsevier, pp. 25-87.

569 Klose, S., Bilen, S., Tabatabai, M.A., Dick, W.A., 2011. Sulfur cycle enzymes, In: Dick, R.P.
570 (Ed.), Methods of Soil Enzymology Soil Science Society of America Madison, WI.

Journal Pre-proof

Journal Pre-proof

571 Klose, S., Wernecke, K.D., Makeschin, F., 2003. Microbial biomass and enzyme activities in
572 coniferous forest soils as affected by lignite-derived deposition. Biology and Fertility of Soils 38,
573 32-44.

574 Lessard, I., Renella, G., Sauvé, S., Deschênes, L., 2013. Metal toxicity assessment in soils
575 using enzymatic activity: Can water be used as a surrogate buffer? Soil Biology and
576 Biochemistry 57, 256-263.

577 López Tomás, L.A., Ordóñez, J.A., de Fernando, G.G., 2006. The p-nitroaniline test to asses
578 the bacterial microbiota of raw ground meat aerobically stored. Meat Science 72, 222-228.

579 Lottenberg, R., Jackson, C.M., 1983. Solution composition dependent variation in extinction
580 coefficients for p-Nitroaniline. Biochimica et Biophysica Acta (BBA) - Protein Structure and
581 Molecular Enzymology 742, 558-564.

582 Margenot, A.J., Nakayama, Y., Parikh, S.J., 2018. Methodological recommendations for
583 optimizing assays of enzyme activities in soil samples. Soil Biology and Biochemistry 125, 350-
584 360.

585 Margenot, A.J., Sommer, R., Mukalama, J., Parikh, S.J., 2017. Biological P cycling is influenced
586 by the form of P fertilizer in an Oxisol. Biology and Fertility of Soils 53, 899–909.

587 Margesin, R., Feller, G., Hämmerle, M., Stegner, U., Schinner, F., 2002. A colorimetric method
588 for the determination of lipase activity in soil. Biotechnology Letters 24, 27-33.

589 Martin, R.M., Wigand, C., Oczkowski, A., Hanson, A., Balogh, S., Branoff, B., Santos, E.,
590 Huertas, E., 2020. Greenhouse gas fluxes of mangrove soils and adjacent coastal waters in an
591 urban, subtropical estuary. Wetlands 40, 1469-1480.

592 Nannipieri, P., Trasar-Cepeda, C., Dick, R.P., 2018. Soil enzyme activity: a brief history and
593 biochemistry as a basis for appropriate interpretations and meta-analysis. Biology and Fertility
594 of Soils 54, 11-19.

595 Parham, J.A., Deng, S.P., 2000. Detection, quantification and characterization of β-
596 glucosaminidase activity in soil. Soil Biology and Biochemistry 32, 1183-1190.

597 Perez de Castro, B., Asensio, M.A., Sanz, B., Ordoñez, J.A., 1988. A method to assess the
598 bacterial content of refrigerated meat. Applied and Environmental Microbiology 54, 1462-1465.

599 Reymond, J.-L., 2004. Spectrophotometric enzyme assays for high-throughput screening. Food
600 Technology and Biotechnology 42, 265-269.

601 Saiya-Cork, K.R., Sinsabaugh, R.L., Zak, D.R., 2002. The effects of long term nitrogen
602 deposition on extracellular enzyme activity in an Acer saccharum forest soil. Soil Biology and
603 Biochemistry 34, 1309-1315.

Journal Pre-proof

Journal Pre-proof

604 Seesom, W., Thongket, P., Yamamoto, T., Takenaka, S., Sakamoto, T., Sukhumsirichart, W.,
605 2017. Purification, characterization, and overexpression of an endo-1,4-β-mannanase from
606 thermotolerant Bacillus sp. SWU60. World Journal of Microbiology and Biotechnology 33, 53.

607 Sinsabaugh, R.L., Gallo, M.E., Lauber, C., Waldrop, M., Zak, D.R., 2005. Extracellular enzyme
608 activities and soil carbon dynamics for northern hardwood forests receiving simulated nitrogen
609 deposition. Biogeochemistry 75, 201-215.

610 Sinsabaugh, R.L., Lauber, C.L., Weintraub, M.N., Ahmed, B., Allison, S.D., Crenshaw, C.,
611 Contosta, A.R., Cusack, D., Frey, S., Gallo, M.E., Gartner, T.B., Hobbie, S.E., Holland, K.,
612 Keeler, B.L., Powers, J.S., Stursova, M., Takacs-Vesbach, C., Waldrop, M.P., Wallenstein,
613 M.D., Zak, D.R., Zeglin, L.H., 2008. Stoichiometry of soil enzyme activity at global scale.
614 Ecology Letters 11, 1252-1264.

615 Sinsabaugh, R.L., Linkins, A.E., 1987. Inhibition of the Trichoderma viride cellulase complex by
616 leaf litter extracts. Soil Biology and Biochemistry 19, 719-725.

617 Sinsabaugh, R.L., Linkins, A.E., 1990. Enzymic and chemical analysis of particulate organic
618 matter from a boreal river. Freshwater Biology 23, 301-309.

619 Stemmer, M., 2004. Multiple-substrate enzyme assays: a useful approach for profiling enzyme
620 activity in soils? Soil Biology and Biochemistry 36, 519-527.

621 Tabatabai, M., 1994. Soil enzymes. Methods of Soil Analysis: Part 2—Microbiological and
622 Biochemical Properties, 775-833.

623 Tabatabai, M.A., Bremner, J.M., 1969. Use of p-nitrophenyl phosphate for assay of soil
624 phosphatase activity. Soil Biology and Biochemistry 1, 301-307.

625 Turner, B.L., 2010. Variation in pH optima of hydrolytic enzyme activities in tropical rain forest
626 soils. Applied and Environmental Microbiology 76, 6485-6493.

627 Wallenius, K., Rita, H., Simpanen, S., Mikkonen, A., Niemi, R.M., 2010. Sample storage for soil
628 enzyme activity and bacterial community profiles. Journal of Microbiological Methods 81, 48-55.

629 Wobbrock, J.O., Findlater, L., Gergle, D., Higgins, J.J., 2011. The aligned rank transform for
630 nonparametric factorial analyses using only anova procedures, Proceedings of the SIGCHI
631 Conference on Human Factors in Computing Systems. Association for Computing Machinery,
632 Vancouver, BC, Canada, pp. 143–146.

633 Yavitt, J.B., Wright, S.J., Wieder, R.K., 2004. Seasonal drought and dry-season irrigation
634 influence leaf-litter nutrients and soil enzymes in a moist, lowland forest in Panama. Austral
635 Ecology 29, 177-188.
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638 **Tables and Figures**

639

640 **Table 1.** Characteristics of the two soils used to furnish high and low enzyme activities.

Soil ID	Soil Series	pH (1:2)	SOC (%)	C:N	CEC (cmol$_c$ kg^{-1})
soil$_{High}$	Flanagan	6.3	3.13	12.7	29.7
soil$_{Low}$	Cisne	7.2	1.43	8.8	11.7

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646 **Table 2.** Description of soil enzymes included and assay methods specific to each corresponding substrate. Abbreviations are used
647 to define the substrate. Footnotes for buffer pH and substrate concentration ([M]) reflect the original publication of these assay
648 parameters used in the present study.

Nutrient Element	Enzyme	*p*NP/*p*NA-linked Substrate	Buffer pH	[S] (mM)	Incubation Time (h)	Bond Type
C	β-glucosidase	*para*-nitrophenyl β-glucopyranoside (**BG**)	6[f]	10[a]	1	Glycosidic
C	β-cellobiohydrolase	*para*-nitrophenyl β-D-cellobioside (**CBH**)	6[g]	2[d,n]	2	Glycosidic
C	β-mannanase	*para*-nitrophenyl a-D-mannopyranoside (**MAN**)	6[a,k]	5[d]	2	Glycosidic
C	β-galactosidase	*para*-nitrophenyl-β-D-galactopyranoside (**GAL**)	6[f]	10[a]	1	Glycosidic
C and N	β-N-acetyl glucosaminidase	*para*-nitrophenyl N-acetyl-β-D-glucosaminide (**NAG**)	5.5[e]	10[c]	1	Glycosidic
N	glycine aminopeptidase	glycine *para*-nitroanilide (**GAP**)	5[b]	2[b]	2	Amide
N	leucine aminopeptidase	leucine *para*-nitroanilide (**LAP**)	5[g,j]	2[d]	1	Amide
P	phosphomonoesterase	*para*-nitrophenyl phosphate (**PME**)	6.5[l]	10[i,m]	1	Ester
P	phosphodiesterase	*bis*-*para*-nitrophenyl phosphate sodium salt (**PDE**)	8[m]	10[h]	1	Ester
S	sulfatase	potassium *para*-nitrophenyl sulfate (**SUL**)	5.8[m]	10[m]	1	Ester

649
650 a. (Acosta-Martínez and Tabatabai, 2000)
651 b. (Allison and Jastrow, 2006)
652 c. (Bailey et al., 2011)
653 d. (Parham and Deng, 2000)
654 e. (DeForest, 2009)
655 f. (Eivazi and Tabatabai, 1988)
656 g. (Hagmann et al., 2015)
657 h. (Margenot et al., 2017)
658 i. (Margenot et al., 2018)
659 j.) (Saiya-Cork et al., 2002)
660 k. (Seesom et al., 2017)
661 l. (Tabatabai and Bremner, 1969)
662 m. (Tabatabai, 1994)
663 n. (Allison, 2008)
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667 **Table 3.** Abiotic hydrolysis of total *p*NP or *p*NA added (%) across four timepoints and under four
668 combinations of matrix type and alkalization base type. Abbreviations are used to define the
669 substrate. Values are mean (n=4).
670

Substrate	**Methods**	**Time (d)**			
		0	**1**	**4**	**7**
BG	Water + Tris	0.0280	0.0308	0.0313	0.0319
	Water + NaOH	0.1675	0.1531	0.1862	0.1741
	Buffer + Tris	0.0279	0.0267	0.0290	0.0314
	Buffer + NaOH	0.4096	0.1347	0.1913	0.2002
CBH	Water + Tris	0.0497	0.0415	0.0850	0.0415
	Water + NaOH	0.3405	0.3568	0.2915	0.4140
	Buffer + Tris	0.0983	0.0779	0.1538	0.0837
	Buffer + NaOH	0.1395	0.4388	0.1210	0.2057
MAN	Water + Tris	0.0258	0.0230	0.0176	0.0203
	Water + NaOH	0.2171	0.1579	0.1466	0.1789
	Buffer + Tris	0.0404	0.0421	0.0362	0.0379
	Buffer + NaOH	0.3839	0.2249	0.2489	0.3839
GAL	Water + Tris	0.0346	0.0335	0.0521	0.0351
	Water + NaOH	0.2177	0.2194	0.2139	0.2045
	Buffer + Tris	0.0355	0.0355	0.0461	0.0343
	Buffer + NaOH	0.6103	0.6009	0.3629	0.3878
NAG	Water + Tris	0.0330	0.0271	0.0319	0.0443
	Water + NaOH	0.0698	0.0681	0.0880	0.1134
	Buffer + Tris	0.0180	0.0157	0.0310	0.0321
	Buffer + NaOH	0.0690	0.0398	0.0726	0.0619
GAP	Water + Tris	0.2803	0.3758	0.3087	0.3190
	Water + NaOH	14.1418	17.1705	16.5669	19.8894
	Buffer + Tris	0.0998	0.1803	0.1136	0.1053
	Buffer + NaOH	34.8977	33.7821	34.5156	32.3528
LAP	Water + Tris	0.7103	0.8083	0.7154	0.7980
	Water + NaOH	28.1089	27.5105	20.7119	24.5613
	Buffer + Tris	0.1884	0.4494	0.2051	0.1995
	Buffer + NaOH	42.7598	41.7494	42.9701	39.3746

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PME	Water + Tris	0.0424	0.0565	0.0467	0.0377
	Water + NaOH	0.1370	0.0881	0.1655	0.0981
	Buffer + Tris	0.0309	0.0245	0.0688	0.0764
	Buffer + NaOH	0.0149	0.0351	0.0727	0.0882
PDE	Water + Tris	0.1765	0.1836	0.1915	0.1971
	Water + NaOH	0.7197	0.7394	0.6214	0.6459
	Buffer + Tris	0.0718	0.1151	0.1172	0.1301
	Buffer + NaOH	0.3061	0.4127	0.5708	0.8495
SUL	Water + Tris	0.0630	0.0543	0.0710	0.0665
	Water + NaOH	0.0870	0.0831	0.1335	0.1191
	Buffer + Tris	0.0611	0.0592	0.0934	0.1175
	Buffer + NaOH	0.1364	0.0669	0.1071	0.1265

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694 **Figure 1.** Three major types of chromogenic substrates used in colorimetric soil enzyme
695 assays, defined by the type of bond hydrolyzed: (a) glycosidic (*para*-nitrophenyl β-D-
696 glucopyranoside), (b) ester (*para*-nitrophenyl sulfate), and (c) amide (L-glycine *para*-
697 nitroanilide). Hydrolysis of glycosidic and ester substrates yields *para*-nitrophenol (*p*NP) and
698 hydrolysis of amide bond substrates yields *para*-nitroanilide (*p*NA).
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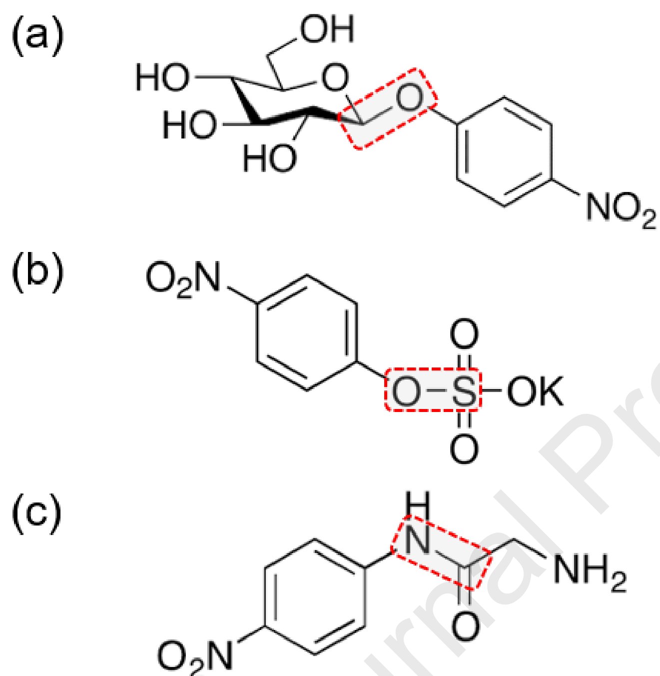

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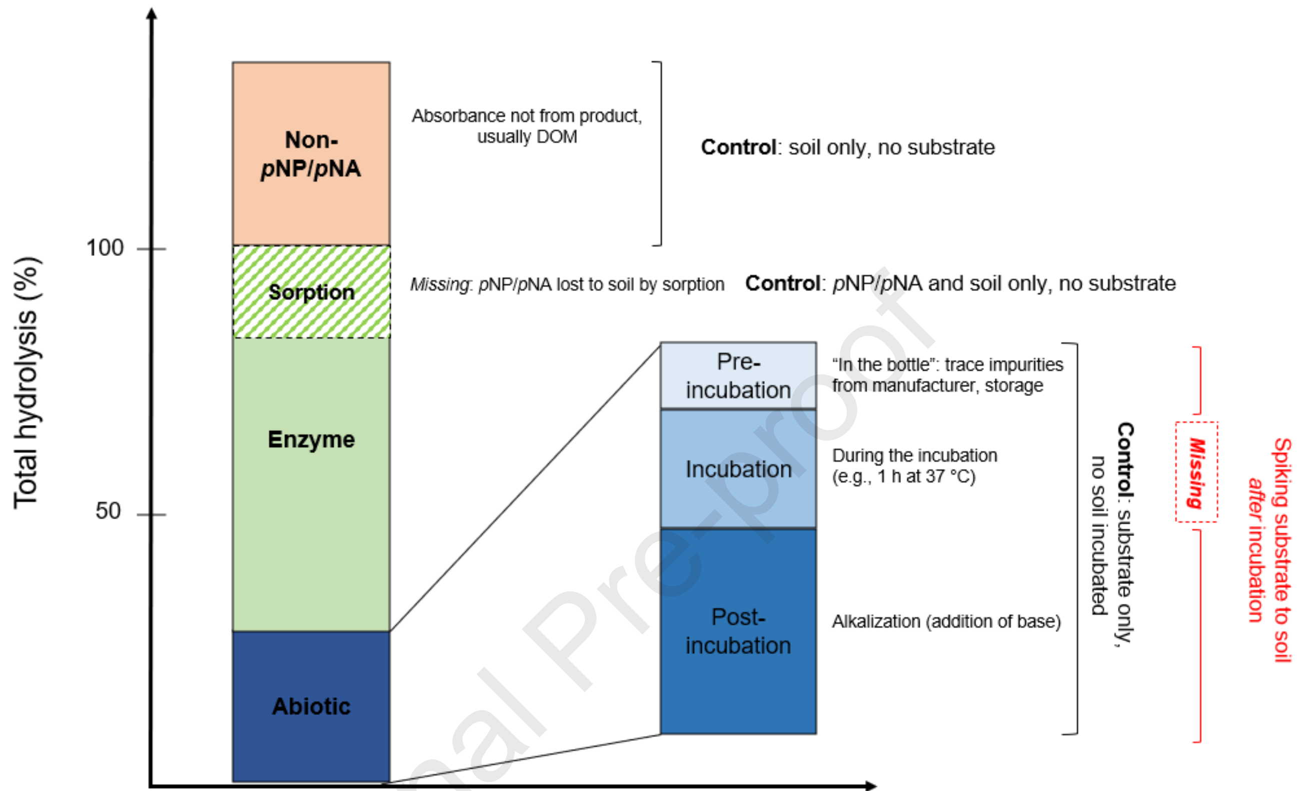
715 **Figure 2.** A conceptual diagram illustrating the components of total hydrolysis of the enzyme
716 substrate, and the recommended controls.



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728 **Figure 3.** Abiotic and total hydrolysis (%) of *p*NP- and *p*NA-linked substrates, for (A) C-cycling enzymes with glycosidic bond
729 substrates, (B) C-/N-cycling enzyme with a glycosidic bond substrate, (C) P- and S-cycling enzymes with ester bond substrates, and
730 (D) N-cycling enzymes with amide bond substrates. Values are mean ± standard error.

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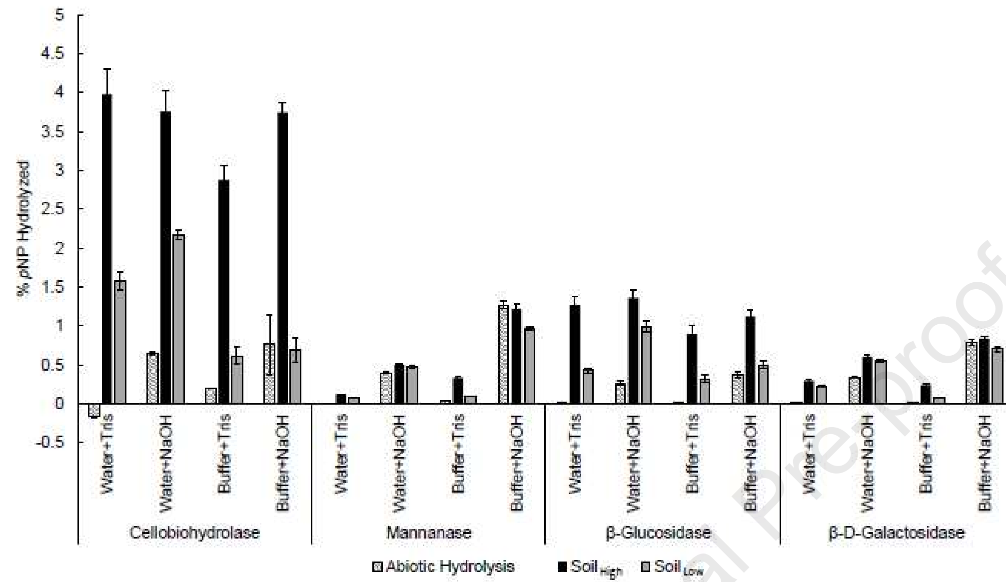

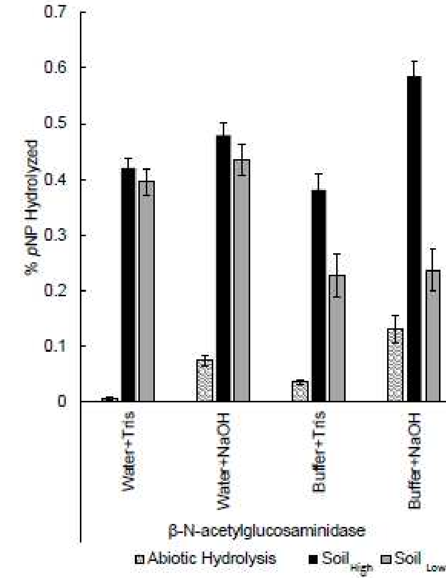

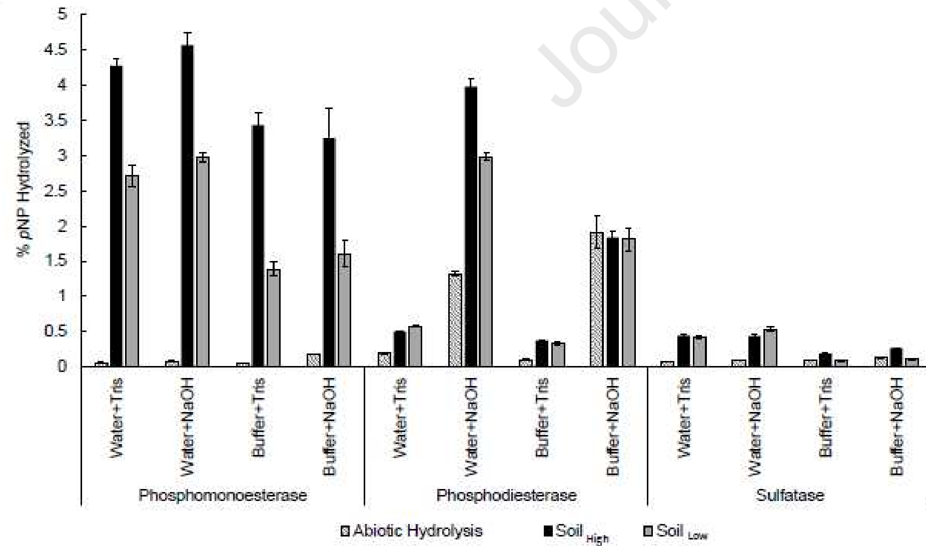

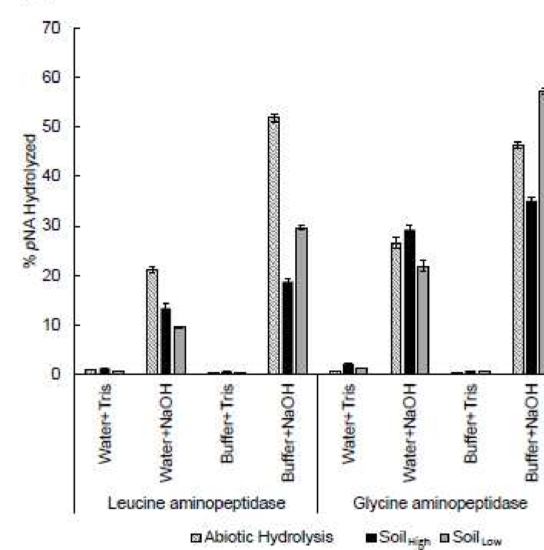

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Title

Sources of abiotic hydrolysis of chromogenic substrates in soil enzyme assays: storage, termination, and incubation

Highlights

- Non-enzymatic (abiotic) hydrolysis of 10 chromogenic substrates evaluated
- Base type in alkalization had a greater effect than matrix and storage duration
- Substrate solutions can be stored for at least 7 days at 4 °C
- Abiotic hydrolysis quantification requires incubation of substrate-only blanks
- Tris, not NaOH, recommended for alkaline termination of *p*NP/*p*NA-based enzyme assays

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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