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Environmental Modeling

Microbial turnover of glyphosate to biomass: utilization as nutrient source, formation of AMPA and biogenic NER in an OECD 308 testAndreas Libonati Brock, Arno Rein, Fabio Polesel, Karolina
Malgorzata Nowak, Matthias Kästner, and Stefan Trapp*Environ. Sci. Technol.*, **Just Accepted Manuscript** • DOI: 10.1021/acs.est.9b01259 • Publication Date (Web): 17 Apr 2019Downloaded from <http://pubs.acs.org> on April 23, 2019**Just Accepted**

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1 **Microbial turnover of glyphosate to biomass: utilization as nutrient**
2 **source, formation of AMPA and biogenic NER in an OECD 308 test**

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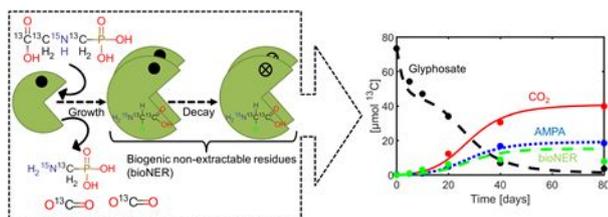
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21 **Abstract**

22 Environmental fate assessment of chemicals involves standardized simulation tests with isotope-
23 labeled molecules to balance transformation, mineralization, and formation of non-extractable
24 residues (NER). Methods to predict microbial turnover and biogenic NER have been developed,
25 having limited use when metabolites accumulate, the chemicals are not the only C source, or
26 provides for other macro-elements. To improve predictive capability, we extended a recently
27 developed method for microbial growth yield estimation for incomplete degradation and multiple-
28 element assimilation and combined it with a dynamic model for fate description in soils and
29 sediments. We evaluated the results against the unique experimental data of $^{13}\text{C}_3$ - ^{15}N -co-labelled
30 glyphosate turnover with AMPA formation in water-sediment systems (OECD 308). Balancing ^{13}C -
31 and ^{15}N - fluxes to biomass, showed a pronounced shift of glyphosate transformation from full
32 mineralization to AMPA formation. This may be explained by various hypotheses, e.g. the limited
33 substrate turnover inherent to the batch conditions of the test system causing microbial starvation
34 or inhibition by P release. Modeling results indicate initial N overload due to the lower C/N ratio in
35 glyphosate compared to average cell composition leading to subsequent C demand and
36 accumulation of AMPA.

37

38 **Keywords:** bound residues, Gibbs Free Energy, microbial growth yield, non-extractable residues,
39 simulation, aminomethylphosphonic acid

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44 **1 Introduction**

45 All chemicals sold commercially in the European Union (EU) require approval under the EU
46 chemicals legislation REACH¹. Standardized simulation tests (e.g. OECD tests 307² and 308³)
47 applying radio- or stable isotope labeled molecules in water, soils, and sediments are used for
48 gaining sufficient information about the general environmental fate and persistence.^{4,5} However,
49 there is still a debate about interpreting OECD 308 tests combining gas-water-sediment interfaces,
50 in particular if the deviation of DegT50 (which is the time until 50% of the parent chemical is
51 degraded) in water and sediments is considered.^{3,6–8} In these tests, the turnover is balanced
52 between mineralization, transformation products and the formation of non-extractable residues
53 (NER).

54 Particular focus for persistence assessment of a chemical is on the formation of NER, which are
55 always formed during such simulation tests,⁹ and can be the largest fraction (up to 90%) of initial
56 label mass at the end of a test.¹⁰ NER are determined by the presence of the isotope label after
57 exhaustive extractions of solid matrices (sediment, soil, sludge, suspended particles etc.) with
58 limited information about their speciation.^{9,11,12} Until recently, neither the potential risk nor the
59 composition of the NER could be reliably determined.⁹ The limited knowledge of the NER
60 speciation resulted in conflicting conclusions regarding the persistence of the active parent
61 chemicals.^{5,13,14} Previous NER definitions have promoted a mismatch between the legislation and
62 the state of knowledge in research and modeling. Only parent compounds and primary metabolites
63 are defined as NER, whereas label conversion to natural bio-components (bioNER), which pose no
64 risk, is explicitly excluded e.g. in the widely accepted definition of Roberts.¹⁵ However, NER
65 assessment based on the remaining isotope labels always include bioNER thus resulting in an
66 overestimation of the potential risks and persistence.^{3,6}

67 Recent advances in analytical methods and theories have helped elucidating the nature and
68 composition of NER and identified bioNER as a major fraction of the formed NER.^{9,11,12,16–19} This
69 has improved the knowledge obtained from OECD tests regarding the potential risks to the
70 environment and human health.⁵ Recently, a method for predicting microbial growth yields of
71 chemicals (Microbial Turnover to Biomass, MTB) was developed²⁰ providing the opportunity to
72 estimate the potential bioNER formation. This method has been applied to estimate bioNER
73 formed from 40 chemicals of environmental concern²¹ and, in addition, to predict input parameters
74 for use in the 'unified model for biodegradation and sorption'.²⁰ With the MTB method, the microbial
75 growth yield can be predicted under the assumption of complete mineralization of the parent
76 compound with productive growth for various terminal electron acceptors (O₂, NO₃⁻, SO₄²⁻).
77 However, metabolites may accumulate diminishing both the matter flux of macro-elements (C, N,
78 P) and the energy gain of the microorganism, eventually resulting in lower growth yields. To date,

79 the MTB method considered the substrate use as C and energy source for the microorganisms
80 while certain substrates may also provide other macro-elements (N, P) at defined stoichiometric
81 ratios. These two factors need to be accounted for in model-based assessment of chemical
82 persistence.

83 Glyphosate is the most widely applied herbicide worldwide²² and is subject of public and scientific
84 debate. Due to its widespread usage, much is known about its fate in the environment for a wide
85 range of conditions in different matrices.^{23–29} Different microorganisms have been isolated capable
86 of using glyphosate as a source of C, N, and P and energy^{24,30–35} but the macro-element relations
87 have never been thoroughly evaluated. Glyphosate is known to be biodegraded via (at least) two
88 pathways, namely the so-called sarcosine pathway and the aminomethylphosphonic acid (AMPA)
89 pathway.^{36–39} In the sarcosine pathway, the C-P bond is cleaved via sarcosine (N-methylglycine)
90 and ortho-phosphate with subsequent complete mineralisation. Sarcosine was never released as
91 metabolite in these experiments but microbial degradation pathways were commonly agreed to
92 occur via this intermediate. However, recent abiotic experiments also showed that glyphosate may
93 directly oxidize in the presence of birnessite to glycine without release of sarcosine.⁴⁰

94 In the AMPA pathway, the C-N bond is cleaved producing AMPA and glyoxylate. AMPA is
95 considered to be the dominating metabolite that accumulates and is frequently detected in soils
96 treated with glyphosate and in adjacent surface waters and sediments.^{24,41–43} Fortunately, the
97 environmental fate of ¹³C and ¹⁵N co-labeled glyphosate in a water-sediment system (OECD 308)
98 was studied recently for the first time,³⁸ and transfer to biomass and bioNER formation was
99 examined by analysis of the dual label incorporation into amino acids hydrolyzed from microbial
100 proteins. In parallel to these processes, also AMPA accumulated. This data set provides the
101 unique opportunity to extend the MTB method combined with the 'unified model for biodegradation
102 and sorption' to multi-element use and incomplete degradation even in multi-phase systems.

103 Therefore, the aim of the present study was to improve predictive capabilities of environmental fate
104 models and to capture these phenomena and to exploit the unique ¹³C and ¹⁵N co-labeled
105 glyphosate data for evaluating the developed combined modeling methods for optimized
106 interpretation of OECD 308 test systems. We aimed at describing metabolite formation (AMPA) as
107 well as energy gain and macro-element fluxes (C and N) into microbial biomass. In addition, we
108 derived and evaluated hypotheses about the metabolite formation and shifts of the metabolic
109 pathways, for example the limitation of microbial growth by other macro-elements than C, here N,
110 which is not mineralized and may cause N overflow in microbial cells. Substrate consumption,
111 formation of products and biomolecules, and distribution of labeled C and N were analyzed to
112 assess metabolic fluxes and macro-element availability.

113

114 2 Materials and Methods

115 **Experimental data.** The authors of the co-labelled glyphosate environmental fate study³⁸ kindly
 116 provided us with their original experimental data for model evaluation. Briefly the analyses included
 117 ¹³CO₂, the extractable ¹³C and ¹⁵N fractions in water and sediment, non-extractable ¹³C and ¹⁵N
 118 fractions in sediment. Amino acids, hydrolyzed from the parent proteins, and their isotopic
 119 composition were analyzed in the living microbial biomass fraction of sediment and in the total
 120 amino acid pool of the sediment fraction (sum of amino acids in both living and dead biomass).
 121 BioNER were quantified from the amount of ¹³C and ¹⁵N in amino acids. Both glyphosate and its
 122 major metabolite AMPA were measured in water and sediment. A schematic overview of the
 123 experimental system is shown in Figure S2a (for more details see Wang et al.³⁸).

124 **Growth yields.** Theoretical microbial growth yields^{20,21} were calculated for glyphosate degradation
 125 via the sarcosine and the AMPA pathways and served as input to the ‘unified model for
 126 biodegradation and sorption’.²⁰ The yield, *Y*, defined as the biomass formed per mass of chemical
 127 consumed, was determined from the Gibbs free energy of the transformation reaction combined
 128 with knowledge of the chemical’s structure and microbial growth processes.²⁰

129 When the mineralization of a chemical requires many steps or is carried out by a multitude of
 130 bacterial strains, the assumption of single-step mineralization may no longer be valid.⁴⁴ The
 131 determination of partial growth yields requires the description of individual metabolic steps and the
 132 flux of macro-elements, energy and electrons within the system must be considered.⁴⁴ The MTB
 133 method can accommodate stepwise transformation by adapting two parameters, namely the
 134 number of electrons, *n*_{bio}, and of C atoms, *n*_C, that can be acquired by microorganisms in a
 135 transformation step. An adjusted MTB method is presented below, with description of partial
 136 growth yield determination.

137 The microbial growth yield is calculated from the anabolic and catabolic yields:

$$\frac{1}{Y} = \frac{1}{Y_{\text{cata}}} + \frac{1}{Y_{\text{ana}}} \quad (1)$$

138 The catabolic yield is determined from the energy of the redox reaction captured by the
 139 microorganisms:

$$Y_{\text{cata}} = \frac{n_{\text{bio}} \Delta G_r^{m'}}{n \Delta G_{\text{ATP}}^{\text{obs}}} \times Y_{\text{ATP}} \quad (2)$$

140 where *n* is the number of electrons transferred in the redox reaction and *n*_{bio} is the number of
 141 electrons from the redox reaction available to the bacterium for energy generation. Empirically, two

142 electrons are transferred for each C-H bond oxidized; n_{bio} thus corresponds to the number of C-H
 143 bonds present in the substrate minus the number of C-H bonds in the formed metabolite. $\Delta G_r^{m'}$ is
 144 the Gibbs free energy of the redox reaction at metabolic conditions (1 mmol L⁻¹ chemical activity,
 145 0.1 mol L⁻¹ ionic strength and pH 7).⁴⁵ $\Delta G_{\text{ATP}}^{\text{obs}}$ is the observed Gibbs energy needed to synthesize
 146 adenosine triphosphate (ATP, approx. 80 kJ mol⁻¹) for typical conditions inside a microbial cell,
 147 calculated from a ΔG value of 31.8 kJ/mol⁴⁶ divided by the microbial efficiency of 40%,⁴⁷ Y_{ATP} is the
 148 biomass yield on ATP (default 5 g cell dw mol⁻¹ ATP for non-sugar substrates).⁴⁷

149 The anabolic yield ($Y_{\text{ana}}^{\text{C}}$) is determined from the amount of C in the substrate available for the
 150 synthesis of new biomass:

$$Y_{\text{ana}}^{\text{C}} = \frac{n_{\text{C}} M_{\text{C}}}{\sigma_{\text{C}}} \quad (3)$$

151 n_{C} is the moles of C acquired by the microorganisms in the transformation [mol], M_{C} is the molar
 152 mass of C [g mol⁻¹], and σ_{C} is the fraction of C in the dry cell [g C_{cell} g⁻¹ cell dw].

153 Furthermore, the anabolic yield can also be dependent on other limiting substrates such as N
 154 ($Y_{\text{ana}}^{\text{N}}$):

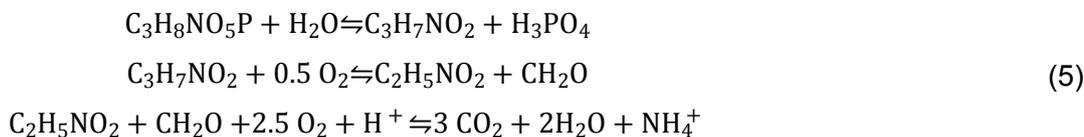
$$Y_{\text{ana}}^{\text{N}} = \frac{n_{\text{N}} M_{\text{N}}}{\sigma_{\text{N}}} \quad (4)$$

155 where the subscript N refers to N. For a microbial cell stoichiometry of C₅H₇O₂N ($n_{\text{C,cell}} = 5$ mol C
 156 per mol cell), 1 mol cell is 113 g (labeled N and C 119 g/mol), σ_{C} is 0.531 g C_{cell} g⁻¹ cell dw and σ_{N}
 157 is 0.124 g N_{cell} g⁻¹ cell dw.⁴⁸

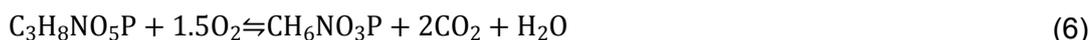
158 **Flux of carbon, nitrogen, energy and electrons.** Glyphosate (C₃H₈NO₅P) is biodegraded via two
 159 pathways: (i) the sarcosine pathway and (ii) the AMPA pathway.^{36–38} All three C atoms and the N
 160 atom can be incorporated into cellular biomass (Figure S1) or released as fully oxidized C in CO₂
 161 and fully reduced N in ammonium (NH₄⁺). The oxidation state of N in glyphosate is -3,
 162 corresponding to its oxidation state in ammonium and amines. The oxidation state of P in GLP is
 163 +3 as it is a phosphonate.⁴⁹ The average oxidation state of the C atoms in GLP is +2/3. Also, when
 164 the phosphonate is oxidized to orthophosphate (+5) two electrons are released and the C in
 165 sarcosine is reduced to an oxidation state of 0. In total, the complete mineralization of glyphosate
 166 releases 12 electrons (see SI S2 for details).

167 **Sarcosine pathway.** Glyphosate is initially transformed into equimolar amounts of sarcosine
 168 (C₃H₇NO₂) and orthophosphate through cleavage of the C-P bond by C-P lyase.³⁶ Sarcosine is
 169 immediately transformed into equimolar quantities of formaldehyde (CH₂O) and glycine (C₂H₅NO₂),

170 in which both labels were found. ^{13}C and ^{15}N co-labelled glycine is thus evidence for the sarcosine
 171 pathway.³⁶ Formaldehyde and glycine are either incorporated into biomass or oxidized to CO_2 . The
 172 full mineralization of glyphosate via the sarcosine pathway can be formulated as:



173 **AMPA pathway.** Glyphosate is oxidized to glyoxylate (CHOCOO^-) and AMPA ($\text{CH}_6\text{NO}_5\text{P}$) through
 174 cleavage of the C-N bond by, e.g., glyphosate oxidoreductase.³⁶ The C in AMPA has an oxidation
 175 state of 0 while the C in glyoxylate has an oxidation state of +2. AMPA and glyoxylate can further
 176 be metabolized to biomass or CO_2 . According to the results of the glyphosate turnover
 177 experiment³⁸, we assume that AMPA is an accumulating metabolite retaining N and P and only C
 178 in glyoxylate is mineralized. The reaction describing the degradation via the AMPA pathway takes
 179 the following form:



180

181 **Partial growth yields, biomass, metabolites and CO_2 formation.** The MTB approach also
 182 includes a C mass balance method to calculate the formation of bioNER.²⁰ A modified method
 183 capable of considering competing transformation pathways, leading to both CO_2 and accumulating
 184 metabolites, is presented below.

185 The moles of C in glyphosate degraded via the AMPA pathway results in the formation of $Y_{\text{AMPA}}^{\text{C}}$
 186 moles of biomass C per mol glyphosate C ($\text{mol C (mol C)}^{-1}$), n_{ox} moles of CO_2 per mol glyphosate
 187 C ($\text{mol C (mol C)}^{-1}$), and C_{AMPA} moles of C in AMPA (mol C):

$$C_{\text{GLP}} = \underbrace{Y_{\text{AMPA}}^{\text{C}} \times C_{\text{GLP}}}_{\text{X formed}} + \underbrace{n_{\text{ox}} \times C_{\text{GLP}}}_{\text{CO}_2 \text{ formed}} + \underbrace{C_{\text{AMPA}}}_{\text{AMPA formed}} \quad (7)$$

188 where C_{GLP} is moles of C in glyphosate. Normalizing with C_{GLP} gives (in units $\text{mol C (mol C)}^{-1}$):

$$1 = Y_{\text{AMPA}}^{\text{C}} + n_{\text{ox}} + \frac{C_{\text{AMPA}}}{C_{\text{GLP}}} = Y_{\text{AMPA}}^{\text{C}} + n_{\text{ox}} + \frac{1}{3} \quad (8)$$

189 Glyphosate degraded via the sarcosine pathway results in the formation of $Y_{\text{SRC}}^{\text{C}}$ moles of biomass
 190 C and $(1 - Y_{\text{SRC}}^{\text{C}})$ moles of CO_2 and the mass balance is simply:

$$C_{\text{GLP}} = \underbrace{Y_{\text{SRC}}^{\text{C}} \times C_{\text{GLP}}}_{\text{X formed}} + \underbrace{(1 - Y_{\text{SRC}}^{\text{C}}) \times C_{\text{GLP}}}_{\text{CO}_2 \text{ formed}} \quad (9)$$

191 One can calculate $Y_{\text{AMPA}}^{\text{C}}$ and $Y_{\text{SRC}}^{\text{C}}$ and the moles of glyphosate degraded via the AMPA pathway
 192 are measured (equal to the moles of AMPA formed), thus the total amount of biomass and CO_2
 193 formed can be found from the sum of biomass and CO_2 formed via both pathways:

$$\text{Total CO}_2 = \underbrace{n_{\text{ox}} \times C_{\text{GLP}}^{\text{AMPA}}}_{\text{AMPA pathway}} + \underbrace{(1 - Y_{\text{SRC}}^{\text{C}}) \times C_{\text{GLP}}^{\text{SAR}}}_{\text{Sarcosine pathway}} \quad (10a)$$

$$\text{Total biomass} = \underbrace{Y_{\text{AMPA}}^{\text{C}} \times C_{\text{GLP}}^{\text{AMPA}}}_{\text{AMPA pathway}} + \underbrace{Y_{\text{SRC}}^{\text{C}} \times C_{\text{GLP}}^{\text{SAR}}}_{\text{Sarcosine pathway}} \quad (10b)$$

194 where $C_{\text{GLP}}^{\text{SAR}}$ and $C_{\text{GLP}}^{\text{AMPA}}$ denote the moles of glyphosate degraded via either pathway. The total
 195 amount of biomass includes both living and dead biomass, i.e., the bioNER formed.

196 **Model structure.** The test system was described as a multi-compartment model including mass
 197 transfer between water and sediment. Based on the experimental results, only glyphosate and
 198 AMPA were explicitly considered in the model, while other intermediates were assumed to be
 199 readily susceptible to biodegradation. State variables were: mass of glyphosate in supernatant
 200 water (W), pore water (D), sediment (A), sequestered (S); mass of AMPA in supernatant water,
 201 pore water, sediment; microbial biomass in sediment (X); mass of CO_2 . The $^{13}\text{C}/^{15}\text{N}$ ratio of the
 202 NER was >3 (except at the final measurement), indicating that the amount of NER formed from
 203 AMPA (C/N equal to 1) was negligible and thus assumed to not occur.

204 The experimental system was unstirred, hence exchanges between compartments were controlled
 205 by diffusive transport.³ Exchange between the supernatant water and the sediment pore water
 206 were described as diffusion through an unstirred boundary layer and to a sediment depth of 1 mm,
 207 based on the calculated depth of diffusion within 80 days⁵⁰ (see also SI S4) and penetration depth
 208 of O_2 .⁷ Biodegradation was assumed to occur only for glyphosate dissolved in the sediment pore
 209 water (D), as negligible formation of AMPA and CO_2 was observed in experiments containing only
 210 the creek water used in the experiment.³⁸ Exchange processes were described with well-
 211 established first-order kinetics,^{11,20} hence, only the equations related to the biodegradation of
 212 glyphosate, microbial growth and formation of bioNER are presented in detail. Calculations were
 213 made in the unit μmol compound. All the model equations can be found in the SI S4, and all input
 214 data are listed in Table S5. A schematic overview of the model compartments is shown in Figure
 215 S2b. Seven model parameters together with the associated model uncertainty were estimated
 216 using the Bayesian optimization method DiffeRential Evolution Adaptive Metropolis algorithm
 217 (DREAM).⁵¹ Details can be found in the SI S5.

218 **Biodegradation of glyphosate.** The experimental data show that glyphosate was a source of both
 219 N and C as ^{13}C and ^{15}N were incorporated into amino acids of proteins, and thus also into microbial
 220 biomass. The N/P ratio in microbial biomass is 13:1 on a molar basis,⁵² while in glyphosate it is 1:1.

221 We can therefore safely assume that N is limiting anabolism before P, unless there are external N
 222 sources available.. The ratio of C/N in microbial biomass is 5:1, while it is 3:1 in glyphosate. Thus,
 223 once N and P supply is secured by degradation of glyphosate via the sarcosine pathway, only C is
 224 limiting microbial anabolism, also because a large fraction of C must be oxidized to CO₂ for the
 225 energy gain (catabolism). Degradation via the AMPA pathway does not provide N nor P for the
 226 degrader but it provides glyoxylate, an excellent C and energy source. Therefore, the observed
 227 shift in pathways, as indicated by the accumulation of AMPA, must be modulated by changes of
 228 the substrate needs. Filling the pools of N or P in the degrading microorganisms combined with
 229 energy limitations may signal the metabolic shift. In order to reflect the experimental results of the
 230 degradation through the AMPA pathway was thus modeled as a being dually limited, i.e. the
 231 respective transformation rate is dependent upon the N from the sarcosine pathway incorporated
 232 into the biomass and down-regulated as long as N is limiting. The metabolic fluxes for glyphosate
 233 degradation through the sarcosine and AMPA pathway are described using *Michaelis-Menten*
 234 kinetics:

$$\frac{dn_{M, SRC}}{dt} = v_{max, SRC} \frac{a_D}{a_D + K_{S, SRC}} X \quad (11)$$

235

$$\frac{dn_{M, AMPA}}{dt} = v_{max, AMPA} \frac{a_D}{a_D + K_{S, AMPA}} \frac{a_D^N}{a_D^N + K_S^N} X \quad (12)$$

236 where n_M [μmol] is the metabolized amount of glyphosate, X is the amount of degrader microbes
 237 [$\mu\text{mol bacteria}$], v_{max} [$\mu\text{mol} (\mu\text{mol bacteria d})^{-1}$] is the maximum transformation rate and K_S [μmol
 238 L^{-1}] is the half-saturation constant for glyphosate through the sarcosine and AMPA pathway
 239 (subscript SRC and AMPA, respectively), and the N released from the sarcosine pathway
 240 (superscript N), a_D is the chemical activity of glyphosate or AMPA (equivalent to the freely
 241 dissolved concentration, $\mu\text{mol L}^{-1}$) in sediment pore water (index D). The chemical activity of
 242 dissolved inorganic N a_D^N is calculated from the NH_4^+ released during mineralization of glyphosate
 243 through the sarcosine pathway resulting in potential turnover inhibition.

244 **Biomass formation.** Microbial growth was described by *Monod* kinetics including a term for
 245 microbial decay (similar to previous studies^{11,53,54}):

$$\frac{dX}{dt} = Y_{SRC} \times \frac{dn_{M, SRC}}{dt} + Y_{AMPA} \times \frac{dn_{M, AMPA}}{dt} - b \times X \quad (13)$$

246 where the first two terms consider microbial growth and the last term considers microbial decay.
 247 Y_{SRC} and Y_{AMPA} [$\mu\text{mol bacteria} (\mu\text{mol substrate})^{-1}$] are microbial growth yields of the sarcosine and

248 AMPA pathways, respectively, $dn_{M,SRC}/dt$ and $dn_{M,AMPA}/dt$ are the metabolic fluxes, and b [d^{-1}] is a
249 first-order rate constant of microbial decay.

250 Microbial necromass, X_{necro} [μmol bacteria], is formed by the decay of living biomass X and is
251 subject to slow mineralization with rate constant k_m [d^{-1}], here set to $0.001 d^{-1}$ comparable to that of
252 soil organic matter:¹¹

$$\frac{dX_{necro}}{dt} = b \times X - k_m \times X_{necro} \quad (14)$$

253 Living bacterial mass and necromass both contribute to the formation bioNER:

$$\frac{dbioNER}{dt} = \frac{dX}{dt} + \frac{dX_{necro}}{dt} \quad (15)$$

254 Microbial growth has been monitored by stable isotope incorporation into amino acids/proteins of
255 living biomass.^{16,17,19,38,55,56} To calculate the total label incorporation into other biomolecules than
256 amino acids, a factor of 2 has usually been applied to calculate the biomass and bioNER resp.,
257 from measured amino acids ($\sim 50\%$ of a dry cell is proteins⁵⁷).⁹

258 **Carbon dioxide.** CO_2 was assumed to originate from the mineralization of glyphosate via both
259 pathways and from microbial necromass:

$$\frac{dn_{CO_2}}{dt} = (1 - Y_{SRC}^C) \frac{dn_{M,SRC}}{dt} n_{C,SRC} + (1 - Y_{AMPA}^C) \frac{dn_{M,AMPA}}{dt} n_{C,AMPA} + k_m X_{necro} n_{C,cell} \quad (16)$$

260 These calculations refer to a C basis: n_{CO_2} [μmol] is the amount of formed CO_2 . Three moles C are
261 metabolized via the sarcosine pathway (with $n_{C,SRC}$ is 3 mol C per mol glyphosate) and only two
262 moles C via the AMPA pathway (with $n_{C,AMPA}$ is 2 mol C per mol glyphosate), since AMPA
263 accumulates. Y_{SRC}^C and Y_{AMPA}^C are microbial growth yields (moles of C in bacteria per moles of C
264 in substrate, or $\text{g C g}^{-1} \text{C}$).²⁰

265

266 3 Results

267 **Microbial growth yield estimates of glyphosate degradation.** Table 1 shows the calculated
268 microbial growth yields and thermodynamic analysis of the different pathways. Negative $\Delta G_r^{m'}$
269 values indicate exothermic, energetically favorable, reactions. The first step of the sarcosine
270 pathway (glyphosate \rightarrow sarcosine) is favorable, while the first step of the AMPA pathway
271 (glyphosate \rightarrow AMPA + glyoxylate) is not. If glyoxylate mineralization is included, the reaction is
272 thermodynamically favorable. $\Delta G_r^{m'}$ of the mineralization via the sarcosine pathway is more than
273 six-fold that of the incomplete AMPA pathway, which is also reflected in the estimated growth
274 yields. The difference between Y_{ana}^C and Y_{ana}^N shows that glyphosate is a better source of N than

275 of C for microbial growth, indicating that C is the limiting substrate. For the sarcosine pathway, the
276 majority of the microbial yield is associated to metabolism of glycine and formaldehyde. The
277 anabolic contribution is higher than the catabolic contribution ($Y_{ana}^C > Y_{cata}$). In the AMPA pathway,
278 most of the potential energy and the N mass are retained in AMPA. If AMPA is an accumulating
279 metabolite (as seen in this study), then only glyoxylate metabolism provides C and energy.
280 However, the question remains open why AMPA is not degraded under the test conditions,
281 although its mineralization seems very energetically favorable, see discussion.

282 **Table 1.** Estimated microbial growth yields of the different degradation steps in the sarcosine and AMPA pathways.

	$\Delta G_r^{m'}$	n_{bio}/n	C-H bonds cleaved	Y_{ana}^N	Y_{ana}^C	Y_{cata}	Y^C	Y^C
	kJ mol^{-1}			g bac mol^{-1}	g bac mol^{-1}	g bac mol^{-1}	g bac mol^{-1}	$\text{g C g}^{-1} \text{ C}$
Sarcosine pathway								
Glyphosate -> sarcosine + P _i	-90	0/0	0	0	0	0	0	0
Sarcosine -> glycine + formaldehyde	-182	2/2	1	0	0	11.4	0	0
Glycine -> 2 CO ₂	-708	4/6	2	119	47.6	29.5	18.2	0.38
Formaldehyde -> CO ₂	-500	4/4	2	0	23.8	31.2	13.5	0.57
AMPA pathway								
Glyphosate -> AMPA + glyoxylate	247	2/2	1	0	0	<0	0	0
AMPA -> CO ₂	-1211	4/6	2	119	23.8	50.5	16.2	0.68
Glyoxylate -> 2 CO ₂	-515	2/4	1	0	47.6	16.1	12.0	0.25
Used for calculation								
Glyphosate -> 3 CO ₂	-1480	8/12	4	119	71.4	61.7	33.1	0.46
Glyphosate -> AMPA + 2 CO ₂	-268	4/6	2	0	47.6	11.2	9.1	0.19

283 The calculations were done for metabolic conditions (superscript m: for chemical activities of 0.1 mmol L⁻¹ and ionic strength of 0.1 mol L⁻¹) and pH is 7
284 (superscript '). $\Delta G_r^{m'}$ is the Gibbs energy of the redox reaction (kJ mol⁻¹), Y_{ana}^C is the anabolic yield on C in the substrate, Y_{ana}^N is the anabolic yield on N
285 in the substrate, Y_{cata} is the catabolic yield gained from the redox reaction, Y^C is the microbial growth yield if C is the limiting substrate and is calculated
286 from Y_{cata} and Y_{ana}^C . Unit is g bacteria dry weight per mol substrate, except the last column in mol C in bacteria per mol C substrate (g C g⁻¹ C). 1 mol
287 bacteria is 119 g (¹³C and ¹⁵N labeled).

288

289 **Model results, uncertainty and parameter identifiability.** The seven calibrated input parameters
 290 and their quality criteria (credibility interval, coefficient of variation σ/μ , maximum absolute
 291 correlation coefficient r) are shown in Table 2. Only $X(0)$ and $K_{OC,GLP}$ can be considered identifiable
 292 based on the criteria by Frutiger et al.⁵⁸ ($r < 0.7$, $CV = \sigma/\mu < 0.5$), due to the high positive
 293 correlation between the v_{max} -values and their accompanying K_S -values.

294 **Table 2:** Result of the DREAM parameter optimization. The set of parameters resulting in the maximum a-
 295 posterior probability are listed together with their 95% credibility interval (CI), the coefficient of variation CV
 296 (σ/μ) and the maximum absolute correlation coefficient (abs r (max)).

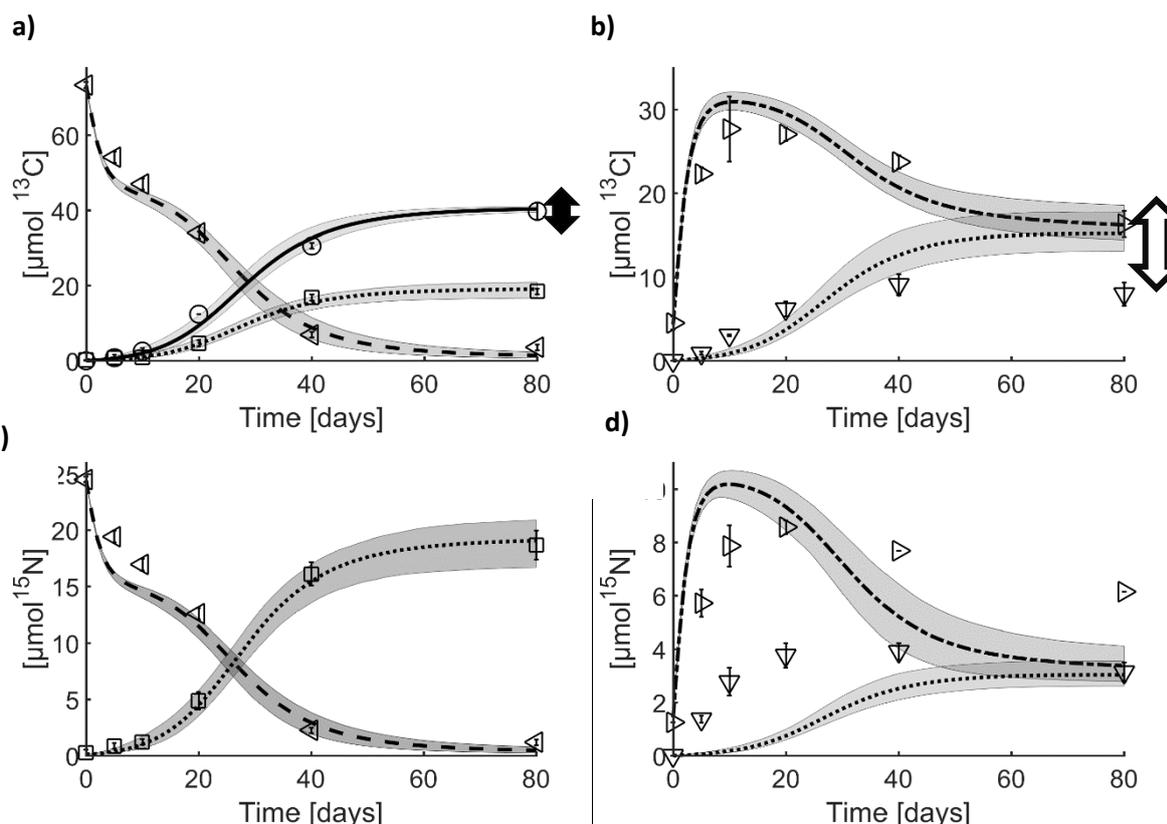
Parameter	Unit	Optimum (95% CI)	CV	abs r (max)
$v_{max, SRC}$	$\mu\text{mol glyphosate } (\mu\text{mol bacteria d})^{-1}$	2.56 (0.47; 3.45)	0.39	0.87
$K_{S, SRC}$	$\mu\text{mol glyphosate L}^{-1}$	391 (52.0; 395)	0.35	0.87
$K_{OC, GLP}$	L kg_{OC}^{-1}	882 (769; 1093)	0.09	0.28
$v_{max, AMPA}$	$\mu\text{mol glyphosate } (\mu\text{mol bacteria d})^{-1}$	26.9 (8.71; 29.6)	0.30	0.89
$K_{S, AMPA}$	$\mu\text{mol glyphosate L}^{-1}$	1327 (431; 1484)	0.29	0.89
$K_{S, N}$	$\mu\text{mol glyphosate L}^{-1}$	47.0 (3.34; 76.9)	0.56	0.50
$X(0)$	$\mu\text{mol bacteria L}^{-1}$	0.050 (0.021; 0.13)	0.45	0.55

297

298 Simulation results for both ^{13}C and ^{15}N are shown and compared to experimental data in Figure 1.
 299 The degradation of glyphosate, formation of AMPA, formation of NER, and formation of CO_2 are
 300 captured very well (RMSE 2.2). Simulated ^{13}C in bioNER was lower than the ^{13}C -total amino acids
 301 until day 40, while simulated ^{15}N in bioNER was strikingly lower than the ^{15}N -total amino acids
 302 measured at all times. Obviously, ^{13}C and ^{15}N were not incorporated into biomass in the expected
 303 C/N ratio of 5:1. The initial difference between bioNER and total NER is three times higher on a ^{13}C
 304 basis than on a ^{15}N basis (Figure 1b,d), and it is likely that it is sequestered glyphosate. In addition,
 305 measured ^{15}N in total amino acids is much higher than the simulated bioNER, indicating that N is
 306 intermediately enriched in the biomass and not released as NH_4^+ , thus N is presumably the control
 307 factor. Remarkably, the use of the simple modified carbon mass balance gives in general similar
 308 final values for bioNER (total biomass) and CO_2 (shown as arrows in Figure 1a,b).

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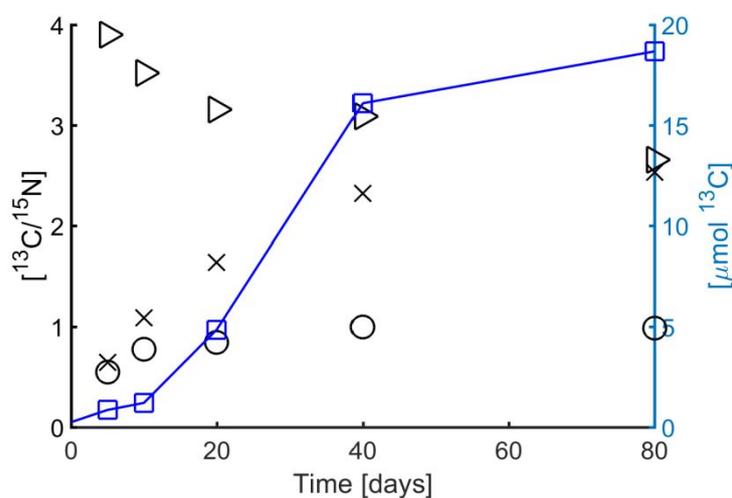
314 **Figure 1.** Model simulations using the optimized parameter set determined using DREAM. Results shown in
 315 $\mu\text{mol }^{13}\text{C}$ (a,b) and $\mu\text{mol }^{15}\text{N}$ (c,d). Lines are model simulations and symbols are measurements. Vertical
 316 bars indicate the standard deviation of the measurements. **(a,c)** Extractable fractions of glyphosate (---; \triangleleft),
 317 AMPA (---; \square) and CO_2 (—; \circ); **(b,d)** Formation of simulated and measured total NER (---; \triangleright), measured
 318 total amino acids (∇) and simulated bioNER (...). The grey bands delineate the 95% credibility interval of
 319 the model simulations. The vertical arrows in panels (a) and (b) show the upper and lower bounds of the
 320 formed amounts of CO_2 (black) and bioNER (white) calculated using the modified MTB carbon balance
 321 method (see SI S8 for details).

322

323 **$^{13}\text{C}/^{15}\text{N}$ -ratio.** Co-labeling with ^{13}C and ^{15}N allowed calculating observed $^{13}\text{C}/^{15}\text{N}$ ratios in amino
 324 acids and NER over the duration of the experiment. The measured $^{13}\text{C}/^{15}\text{N}$ ratios in total amino
 325 acids, NER, and amino acids in living cells are shown in Figure 2 together with the formation of
 326 AMPA. The $^{13}\text{C}/^{15}\text{N}$ ratios in amino acids in living cells and total amino acids were initially <1 , with
 327 the ratio in amino acids in living cells converging to approximately 1 at the end of the experiment.
 328 The $^{13}\text{C}/^{15}\text{N}$ -ratio in total amino acids increased concomitantly with the formation of AMPA to a
 329 maximum of 2.6, while the average C/N ratio of amino acids in living cells is 3.7:1 (Table S2). This

330 shows that ^{15}N was initially incorporated into amino acids to a larger extent than ^{13}C . As glyphosate
331 is an aminophosphonic acid analog of glycine, it is not surprising that ^{13}C and ^{15}N were
332 predominately found in glycine (60% of the ^{13}C and 34% of the ^{15}N measured in total amino acids
333 on day 5).³⁸ The Y^{C} for the sarcosine pathway (Table 1) is 0.46, i.e. 54% of C forms CO_2 if
334 glyphosate is the sole C source. However, the measured $^{13}\text{CO}_2/^{13}\text{C}$ -total amino acids ratio was <1
335 until day 20 (Fig. 2). After day 20, when the AMPA pathway dominates, only ^{13}C was incorporated
336 in amino acids, and the $^{13}\text{C}/^{15}\text{N}$ -ratio in total amino acids rose above 1. In the NER, the $^{13}\text{C}/^{15}\text{N}$ -
337 ratio was >3 in all measurements except the last (80 days). At the end of the experiment, the
338 $^{13}\text{C}/^{15}\text{N}$ ratios in NER and in total amino acids were similar, suggesting that the NER are
339 predominately biogenic, which is supported by the model simulations. Therefore, the modeling
340 approach reflecting the experimental data conclusively shows that C is limiting or the excess of N
341 is triggering the shift from sarcosine pathway towards AMPA formation.

342



343

344 **Figure 2.** $^{13}\text{C}/^{15}\text{N}$ ratio measured in the experiment. Left axis: $^{13}\text{C}/^{15}\text{N}$ ratio in the measured total amino acids
345 (\times), living amino acids (\circ), and non-extractable residues (NER) (\triangleright) over time. Right axis (blue): Measured
346 AMPA in μmol (\square).

347

348

349 4 Discussion

350

351 The primary goal of the present study was to extend and validate the microbial growth yield
352 estimation (MTB) method combined with the ‘unified model for biodegradation and sorption’ in
353 order to capture the phenomena of various transformation pathways with metabolite formation and
354 multi-substrate use for optimized interpretation of OECD 308 (and also 307) test systems. This was
355 performed for a ^{13}C and ^{15}N co-labeled glyphosate degradation experiment³⁸. Glyphosate
356 consumption, formation of metabolites (AMPA) and biomolecules, as well as energy gain and
357 distribution of ^{13}C and ^{15}N were successfully modeled and the metabolic fluxes and element
358 availability were assessed. Anabolic yield calculations conditioned on the microbial C and N
359 demands gave insights into nutrient limitations and were confirmed by the measured $^{13}\text{C}/^{15}\text{N}$ ratios.
360 The results showed that glyphosate mineralization via the sarcosine pathway gave a higher growth
361 yield than via the (incomplete) AMPA pathway. However, in a later stage of degradation the
362 release of AMPA may protect the cells from N overflow which may cause the accumulation of this
363 transformation product. The extended modeling methods allow improved interpretations and
364 hypothesis derivation for transformation pathways in environmental fate test systems by
365 considering the bioenergetic feasibility and influential factors, such as nutrient limitation and
366 element distribution.

367 **Model performance.** The model simulations were able to fully capture the experimental
368 elimination of glyphosate and the concurrent formation of total NER, AMPA and CO_2 observed in
369 the OECD 308 setup. However, the formation of biomass as observed by amino acid analysis was
370 only partly reflected. This is caused by the analytical bias introduced when biomass formation is
371 calculated from amino acid analysis of hydrolyzed microbial proteins (SI S3). While other model
372 structures^{3,6,8} have been used to capture the dynamics of the OECD 308 test system and formation
373 of NER they do not provide any information regarding the biomass formation, NER composition,
374 and macro-element distribution. The prediction of the NER composition, in particular the bioNER
375 contribution, requires the mechanistic description of microbial growth and decay in the model.

376 The calibration procedure resulted in acceptable uncertainty ranges of the estimated parameters
377 and model output (Table 2 and Figure 1). The maximum specific growth rate found for the AMPA
378 pathway ($\mu_{\text{max, AMPA}}$ 2.04 d^{-1}) is comparable to previously reported findings, while, the one
379 determined for the sarcosine pathway ($\mu_{\text{max, SRC}}$ 0.71 d^{-1}) is lower.³⁶ However, direct comparison is
380 difficult due to the difference in the measurement units reported. The determined affinity constant
381 $K_{\text{S, SRC}}$ (391 $\mu\text{mol L}^{-1}$) is higher than the ranges reported for *Pseudomonas* sp. strain PG2982 (23
382 $\mu\text{mol L}^{-1}$) and *Arthrobacter* spp. (105–125 $\mu\text{mol L}^{-1}$).^{32,59} For soil microcosms the value was
383 estimated to be 412–4050 $\mu\text{mol L}^{-1}$.⁶⁰ The value obtained for $K_{\text{S, N}}$ is within the affinity data
384 compiled for ammonia elsewhere.⁶¹

385 **Shift of transformation pathways.** From a thermodynamic perspective, the sarcosine pathway is
386 preferable to the AMPA pathway (Table 1) and gives access to the nutrients N and P, although in
387 an over-stoichiometric relation in comparison to microbial biomass. However, the experimental
388 data show that microorganisms favored the AMPA pathway after day 10. Based on the macro-
389 element availability in glyphosate, we hypothesized that P is in surplus in this molecule and that N
390 saturation and C deficit modulated the switch of pathways leading to the formation of AMPA. The
391 calculated Y_{ana}^N is 1.7 times higher than Y_{ana}^C , further indicating that C is limiting the anabolism.
392 Microorganisms thus need to support their growth by using other C sources than glyphosate, since
393 the glyphosate degradation via the sarcosine pathway forms CO_2 resulting in NH_4^+ release and
394 overflow in the living cells. Once sufficient N is available from the sarcosine pathway due to C
395 mineralisation, the faster formation of AMPA dominates and prevents the cells from internal
396 ammonia-N overflow. This may be an explanation for the AMPA accumulation under the batch
397 conditions of the OECD test 308 and other batch test systems and also explain AMPA occurrence
398 in the environment due to slower AMPA turnover.⁶² Similar results were found for glyphosate in
399 OECD 307 fate studies in soil but with lower amounts of microbial biomass.⁶³ Also, the sarcosine
400 pathway is more costly in terms of enzyme synthesis (C-P lyase), which may cause slower
401 substrate turnover and growth of microorganisms than the AMPA pathway (see v_{max} and μ_{max}
402 values, Table 2 and Table S5).

403 The $^{13}C/^{15}N$ ratio measured in amino acids is much lower than 3:1 (in glyphosate) until day 40,
404 which indicates the use of non-glyphosate C sources for anabolism and challenges the assumption
405 of single substrate use and stable isotope probing approaches in general.⁶⁴ Under the batch
406 conditions of OECD 308 test, but presumably also in soils (OECD 307), the initial mixing and
407 rewetting of the sediment can make organic matter available as substrate and lead to an initial
408 burst of (non-labeled) CO_2 (Birch effect).⁶⁵ When this initial effect is gone, starvation will prevail and
409 may also trigger a shift in the glyphosate degradation pathways, particularly under C limitations.
410 The use of other C sources without a considerable impact on catabolism or anabolism may be
411 explained by the mining of microbial building blocks by the degrader microorganisms with minimal
412 energetic impact on the anabolism. These building blocks can be derived from microbial
413 necromass always present in sediments and soils. Additional C sources have consequences for
414 the amount of living biomass when calibrated to measured amino acids, and for the fitted maximum
415 rate ($v_{max} \cdot X$). We therefore excluded the measurements of amino acids hydrolyzed from proteins
416 in the model calibration. However, these were used to assess biomass and bioNER formation. In
417 SI S3, theoretically sound conversion factors are derived to convert measurements of ^{13}C - or ^{15}N -
418 amino acids into total biomass. “Apparent” conversion factors can be calculated by dividing the
419 simulated ^{13}C - or ^{15}N -bacterial biomass with the measured ^{13}C - or ^{15}N -total amino acids (Figure 1).

420 The "apparent" conversion factors not only varied in time, they were also much lower than the
421 theoretical factors for glyphosate as sole substrate derived in SI S3 and the commonly applied
422 factor of 2.¹² For N, the apparent factor was as low as 0.16 and increased to 0.97 by the end of the
423 experiment. For C it increased from 0.33 to 1.9. This further indicates that N is predominately used
424 in amino acid synthesis and is stored within the cells whereas C is not. Consequently, the
425 theoretical conversion factors and the commonly applied factor of 2 are not valid under the
426 observed conditions. Consequently, the use of these leads to the overestimation of bioNER early in
427 the simulation test.

428 Hypothetically, the shift in transformation pathways could be caused by both N and P releases.
429 Low intracellular orthophosphate (P_i) concentrations have been shown to enhance the activity of
430 the enzymatic complex (C-P lyase) of the sarcosine pathway while high concentrations are
431 inhibitory.^{35,37} The maximum concentration of P_i in the top 1 mm pore water (= boundary layer,
432 assuming that 90% of all glyphosate- P_i is released)⁵⁹ and overlying water is $78 \mu\text{mol L}^{-1}$, which is
433 similar to the inhibition coefficients (K_i) reported ($24\text{--}253 \mu\text{mol L}^{-1}$) and may explain the inhibition
434 of the sarcosine pathway.^{31,60} The concentration is however much lower than the inhibition
435 coefficient reported for the mutant strain GLP-1/Nit-1 ($2,300 \mu\text{mol L}^{-1}$).³¹ In addition, P_i competes
436 with glyphosate for sorption sites, indicating that it does not remain in solution⁶⁶ and thus the P
437 inhibition hypothesis in the present experiments appears unlikely. This hypothesis together with the
438 hypothesis that AMPA is degraded has been tested using the model and further information can be
439 found in SI S6. In Lake Greifensee in Switzerland, the concentration of both glyphosate and AMPA
440 was observed to decline concomitantly with the depletion of P_i and a bloom of cyanobacteria.⁴²
441 Cyanobacteria are photoautotrophs, hence, unaffected by the lack of an organic C source. As
442 some species are also capable of fixing atmospheric N, it is likely that the disappearance of AMPA
443 and glyphosate in such lakes is driven by a need for P.

444 Considering the C limitation and the less likely impact of P inhibition, N can be identified as the
445 trigger factor of the shift of degradation pathways to AMPA accumulation. Via the sarcosine
446 pathway, C is mineralized and eliminated whereas N is not. As shown by the experimental data,
447 NH_4^+ obviously remains in the degrader cells thus leading to an N overflow within the cells. Thus, it
448 is justified to hypothesize that the accumulation of AMPA provides a solution to discard excess N
449 and P and at the same time provide glyoxylate as a C and energy source.

450 **Relevance and research needs.** The 'unified model for biodegradation and sorption' combined
451 with the extended MTB approach provides a powerful tool for the simulation of biodegradation and
452 bioNER formation, even in complex experimental settings and with multiple pathways and macro-
453 element availability. We could show that the developed modeling approach was able to capture

454 water-sediment mass transfer as well as turnover of real experimental data based on yield
455 estimates for incomplete metabolism. When combined with experiments with multiple isotope
456 labels in a single substrate,³⁸ the anabolic yield calculations gave valuable additional insights, in
457 particular when compared to measured ¹⁵N and ¹³C labels, and provided the unique opportunity to
458 derive hypotheses to explain the shift of microbial degradation and metabolite formation pathways.
459 The results show that glyphosate degradation is C limited and increasing internal overflow of
460 ammonia in degrader cells, presumably combined with starvation of the C turnover under the test
461 conditions, may cause the accumulation of AMPA. One may speculate that in order to avoid AMPA
462 accumulation when using glyphosate, application of fertilizer and N-rich manure should be applied
463 only when easily assimilable C sources are available or should be supplied much later than
464 glyphosate application. Future studies should therefore investigate factors determining the
465 accumulation of AMPA (being a potential nutritious substrate) for mitigating its occurrence as a
466 widely observed accumulating metabolite of a generally biodegradable herbicide.

467

468

469 **Supporting Information**

470 Data for the calculation of the microbial growth yields; half-reactions describing the transformation
471 pathways; equations and tables showing how the factors for converting C and N amounts in amino
472 acids to C and N amounts in bacterial cells were derived; equations for the model implementation;
473 detailed results of the DREAM parameter inference method; C and N balances.

474

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