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1 Experimental evidence that clay inhibits bacterial decomposers,
2 with implications for the preservation of organic fossils

3

4 **Sean McMahon, Ross P. Anderson, Erin E. Saupe, and Derek E. G. Briggs**

5 Department of Geology and Geophysics, Yale University, 210 Whitney Avenue, New
6 Haven, CT 06511, USA

7

8 **ABSTRACT**

9

10 Exceptionally preserved organic fossils are commonly associated with clay-rich horizons
11 or directly with clay minerals. It has been posited that interactions between clay minerals
12 and organic tissues inhibit enzymatic reactions or protect carcasses in such a way that
13 decay is inhibited. However, interactions between clay minerals and the biological agents
14 of decay, especially bacteria, may be at least as important to preservation potential. Here
15 we show that clays of particle size $< 2 \mu\text{m}$ in suspensions exceeding 10 mg/ml in
16 concentration inhibit the growth of *Pseudoalteromonas luteoviolacea*, a marine
17 heterotrophic bacterium involved in the decay of marine animals. Such clay–microbe
18 interactions can contribute to exceptional preservation and specific examples may play a
19 role in shaping the distribution of Konservat-Lagerstätten through time.

20

21 INTRODUCTION

22

23 Exceptionally preserved fossil assemblages (Konservat-Lagerstätten *sensu* Seilacher,
24 1970) that yield the remains of soft tissues constitute an invaluable source of information
25 about the history of life on Earth. In diverse lagerstätten of all ages, organic remains are
26 associated with aluminosilicate clay minerals (e.g., Butterfield, 1994; Anderson et al.
27 2011; Laflamme et al., 2011; Cai et al., 2012; Pan et al., 2014; Wacey et al., 2014). A
28 large and paleobiologically important subset of these lagerstätten exhibit Burgess-Shale
29 type (BST) preservation, in which organic material is retained and flattened parallel to
30 bedding in fine-grained siliciclastic lithologies often with high clay-to-organic ratios
31 (Butterfield, 1990; Butterfield et al., 1994, Butterfield, 1995; Gaines, 2014). Indeed this
32 is the principal mode of preservation for eukaryotic remains in Proterozoic successions
33 (e.g. Cohen and Macdonald, 2015; Yuan et al., 2011; Butterfield et al., 1994), as well as
34 soft-bodied Cambrian marine metazoan faunas (e.g., Butterfield, 1990; Gaines et al.,
35 2008; Gaines 2014). Many workers have argued that temporal and environmental
36 distributions of clay minerals impart patterns and biases to the fossil record (e.g.
37 Butterfield, 1995). Butterfield (1990) attributed the preservation of organic material to
38 the effect of clays in adsorbing and deactivating autolytic enzymes, Orr et al. (1998)
39 proposed that early diagenetic clays templated original organic tissues, and Petrovich
40 (2001) suggested that Fe²⁺ ions stabilized the tissues and acted as nucleation sites for clay
41 minerals. Wilson and Butterfield (2014) proposed that Al³⁺ ions derived from a clay-rich
42 substrate protected the tissues from decay in a process analogous to tanning and
43 speculated that Fe-rich clays could have a similar effect. Independent of the association

44 of clays and fossils, there is abundant evidence that clay minerals interfere with bacterial
45 growth (e.g. Wong et al., 2004; Williams et al., 2011; Morrison et al., 2016).

46 We tested the hypothesis that clay mineral particles inhibit the growth of bacterial
47 decomposers by comparing the growth of *Pseudoalteromonas luteoviolacea* (a
48 heterotrophic marine γ -proteobacterium) in the presence of various clay minerals,
49 calcite, and in a mineral-free control. Bacteria of this widespread genus are commonly
50 found in biofilms associated with live marine animals, have the capacity to degrade a
51 range of tissue types, and constitute a large proportion of the bacterial assemblage in
52 submerged carcasses (Skovhus et al., 2007; Raff et al., 2008; Dickson et al., 2011). The
53 strain used here was shown to decompose sea urchin embryos *in vitro* (Raff et al., 2013).

54

55 **MATERIALS AND METHODS**

56

57 Five minerals were used in the experiments: berthierine (Scarborough, UK; Yale Peabody
58 Museum (YPM) MIN 100586), calcite (Budapest, Hungary; YPM MIN 056322),
59 glauconite (Odessa, Delaware; YPM MIN 043086), illite (Silver Hill, Montana; Clay
60 Minerals Society #IMt-2), and kaolinite (Santa Cruz Biotechnology Company, USA).
61 Specimens were rinsed in distilled water, ground with an agate mill and sonicated to
62 obtain fine powders, which were then rinsed again by centrifuging in distilled water at
63 $2773 \times g$ (4000 rpm) for 15 minutes, three times. Following 10 minutes of sonication to
64 disaggregate particles, grains of up to two microns in diameter were obtained by
65 centrifuging in distilled water at $97 \times g$ (750 rpm) for 5 minutes, discarding the pellet and
66 retaining the supernatant, which was removed and dried at 45 °C. This ensured that all
67 minerals were similar in particle size to natural mud and to each other.

68 Bacterial growth medium (Difco Marine Broth [MB]) was prepared in sterile
69 conditions, filtered to remove particles >20 µm, and added in aliquots of 950 µl to glass
70 culture vials (8 ml; Wheaton/Fisher Scientific) to which the clays were added
71 immediately prior to autoclaving. Additional sonication was employed to disaggregate
72 mineral particles prior to inoculation. Vials were prepared with suspended mineral
73 concentrations of 5, 10 and 25 mg/ml (Fig. 1) for each of the five mineral species and for
74 a control with no suspended mineral particles.

75 The inoculum was taken from a stock culture on agar plates of *Pseudoalteromonas*
76 *luteoviolacea* (ATCC33492) and grown in MB to an optical density (OD₆₀₀) of ~0.5
77 (above sterile MB). Vortex-mixed aliquots of 50 µl were transferred under sterile
78 conditions to each of the clay-bearing vials, which were incubated on a rotary shaker at
79 25 °C. The use of continuously agitated suspensions rather than settled sediment ensured
80 thorough and homogeneous mixing of mineral particles and bacteria. Preliminary results
81 suggested that the presence of mineral particles interfered with normal techniques for
82 measuring bacterial growth. We therefore subsampled the experimental cultures (post
83 vortexing) after 24 hours' growth and diluted them in fresh medium so that both clay
84 particles and bacteria were 100 times less concentrated. The bacteria multiplied in the
85 fresh medium so that geometric differences in population size were inherited from the
86 original cultures, while clay concentration remained minimal. Turbidity at 600 nm (a
87 standard proxy for bacterial cell density) was measured using a spectrophotometer (Hach
88 DR/2010) in mid-exponential phase (after six hours of growth), at which time the
89 disparity in population sizes was clearly evident. Initial values were subtracted so that
90 any turbidity due to clay was excluded. All experiments were performed in
91 quadruplicate.

92 Statistical analyses were performed using the R programming language (R Core
93 Team, 2016). For each mineral concentration turbidity was normalized by the mean of
94 the clay-free control treatment. Using the control-standardized data, two-way analyses of
95 variance (ANOVA, Type III Sum of Squares) were performed on turbidity levels in the
96 ‘car’ R package (Fox and Weisberg, 2011), with mineral concentration and mineral
97 species as factors. Planned post-hoc comparisons were performed in the phia R package
98 (De Rosario-Martinez, 2013) using the Holm (1979) correction for multiple comparisons.
99 Such pair-wise comparisons were made to test for statistical differences between factor
100 levels (e.g., kaolinite versus control) and were made independently both within and
101 across the three mineral concentrations. Effect sizes were calculated using the etaSquared
102 function in the lsr R package (Navarro, 2015), which reveals the proportion of the
103 variance in the dependent variable that is attributable to the factor in question. We also
104 examined the effect of mineral species and mineral concentration on bacterial growth by
105 considering the four clay minerals (berthierine, glauconite, illite, and kaolinite) as a
106 single category. The above analyses were repeated using this binned dataset. All data
107 were normally distributed, homoscedastic, and showed linear structure based on Q-Q,
108 scale-location, and residuals vs. fitted values plots, respectively.

109

110 **RESULTS**

111

112 A two-factor analysis of variance showed significant main effects of mineral
113 concentration, $F(2, 53) = 58.3, p < 0.05 (\eta^2 = 0.40)$ and mineral species, $F(5, 53) = 2.9, p$
114 $< 0.05 (\eta^2 = 0.29)$ on turbidity, but these effects were qualified by an interaction between

115 the two variables, $F(10, 53) = 11.56$, $p < 0.05$ ($\eta^2 = 0.20$) (Supplementary Table 1),
116 discussed below.

117 Post-hoc comparison tests revealed no significant difference in bacterial growth
118 among mineral species compared to the control at 5 mg/ml, with the exception of
119 glauconite (Fig. 1A; Supplementary Table 2). The significance of the response to
120 glauconite at 5 mg/ml suggests an unusually low minimum inhibitory concentration with
121 just a slightly increased effect at higher concentrations. Mineral suspensions of 10 and 25
122 mg/ml resulted in significantly depressed bacterial growth for all mineral species
123 compared to the clay-free control, with the exception of calcite (Figs. 1A and 1B). The
124 response at 25 mg/ml is much more pronounced to berthierine and kaolinite than to the
125 other clay minerals—a fourfold decrease in turbidity compared to the control and
126 threefold compared to calcite. We observed an inverse relationship between mineral
127 concentration and turbidity for all mineral species except calcite, but the strength and
128 significance of this relationship varies with mineral species (Supplementary Table 3).
129 Although calcite appears to impede bacterial growth more strongly at 10 mg/ml than at
130 25 mg/ml, this is not significant.

131 In order to examine the impact of clays in general, we compared the effect of the
132 clay minerals as a group with that of calcite and the control (Fig. 2). The two-factor
133 analysis of variance showed significant main effects of mineral concentration, $F(2, 62) =$
134 5.98 , $p < 0.05$ ($\eta^2 = 0.40$) and mineral species, $F(2, 62) = 1.23$, $p < 0.05$ ($\eta^2 = 0.30$) on
135 turbidity, qualified by their interaction, $F(4, 62) = 12.246$, $p < 0.05$ ($\eta^2 = 0.13$)
136 (Supplementary Table 1). Post hoc comparisons revealed no significant effect of clay or
137 calcite on bacterial growth at 5 mg/ml. Both clay and calcite minerals, however, affected
138 growth significantly at 10 and 25 mg/ml, but in both instances clay minerals were

139 associated with significantly less growth than calcite (Supplementary Table 2). Increasing
140 clay concentration resulted in significantly depressed bacterial growth, but the same was
141 not true for increasing calcite concentration (Supplementary Table 3). The marginal
142 means and standard deviations of all within-concentration analyses are presented in
143 Supplementary Table 4.

144 In summary, the results of our experiments show that the growth of
145 *Pseudoalteromonas luteoviolacea*, a marine heterotrophic bacterium known to degrade
146 animal tissues, is significantly inhibited by the presence of clay-sized mineral particles.
147 With increasing mineral concentration, this inhibition becomes more pronounced and
148 shows greater variation between mineral species. Clay minerals produce a stronger effect
149 than calcite particles of similar size.

150

151 **DISCUSSION AND CONCLUSIONS**

152

153 Our experimental results support the hypothesis that clay minerals impede the growth of
154 bacterial decomposers, providing a way to facilitate the preservation of soft tissues.
155 Hypotheses to explain particular instances of exceptional fossil preservation have
156 implicated both detrital clays, and authigenic clays that formed in sedimentary pore
157 spaces and in association with decaying organic matter (e.g., Butterfield, 1990; Orr et al.
158 1998; Gabbott et al. 2001). Our results are relevant in both cases, and furthermore they
159 suggest that certain clays are more likely to inhibit bacterial activity than others.

160 Our results are consistent with the usually high clay content of Cambrian Burgess
161 Shale-type fossil-hosting sediments (Curtin and Gaines, 2011; Forchielli et al., 2014;
162 Gaines et al., 2011; Powell, 2003). However, there is currently limited documentation of

163 associations with specific clay compositions. An exception is the Mount Cap Formation,
164 where glauconite is associated with fossiliferous mudrock horizons (Aitken et al., 1973;
165 Butterfield, 1994). Indeed, macrostratigraphic data from Laurentia reveal a spike in
166 glauconite production during the Cambrian (Peters and Gaines, 2012). More generally, it
167 has been argued that the rise and fall of BST preservation tracked the weathering flux of
168 Al-rich clays as well as particular biogeochemical conditions that affected the
169 composition of clay in marine sediment (Butterfield, 1995; Wilson & Butterfield 2014).
170 Preliminary data through a Cryogenian succession in Mongolia provide a further example
171 of clays of specific composition (in this case berthierine) in fossiliferous horizons
172 (Anderson et al., 2014).

173 In both BST and non-BST lagerstätten, clay minerals can also be found intimately
174 associated with organic fossils, often coating their external surfaces (e.g. Gabbott, 1998;
175 Orr et al., 1998; Gabbott et al., 2001; Anderson et al., 2011; Laflamme et al., 2011; Cai et
176 al., 2012; Pan et al., 2014; Wacey et al., 2014). A more refined mineralogical
177 characterisation of clays within fossiliferous laminae and surrounding individual fossils
178 awaits the application of emerging microscopic techniques (Tosca et al., 2015).

179 Our results are also consistent with the higher quality of organic tissue preservation
180 associated with clay minerals as opposed to carbonate throughout the geological record.
181 Fine-grained carbonates tend to preserve organic tissues more rarely and with less fidelity
182 than clays (e.g., Butterfield et al., 1994). Organic fossils in Proterozoic carbonates, for
183 example, tend to be dominated by robust testate forms (e.g., Bosak et al., 2011a; Bosak et
184 al., 2011b; Bosak et al., 2012; Dalton et al., 2013) in contrast to more delicate forms
185 preserved in clay-rich shales (e.g. Butterfield et al., 1994; Butterfield & Rainbird, 1998).

186 However, other factors may contribute to these differences, such as the higher
187 sedimentation rate represented by clastic beds (Canfield, 1994).

188 The antibacterial properties of clays are attributed mostly to the toxicity of metal
189 cations, particularly Al^{3+} and Fe^{2+} (e.g., Wong et al., 2004; Morrison et al., 2016).
190 Diverse bacteria are susceptible to Al^{3+} , while excessive Fe^{2+} in aerobic conditions causes
191 oxidative damage to bacterial cells (Guida et al., 1991; Kapoor and Arora, 1998;
192 Amonette et al., 2003; Imlay et al., 2008). It is therefore striking that kaolinite and
193 berthierine, the most aluminum-rich and ferrous-iron-rich clays in our experiments
194 respectively, were associated with the strongest suppression of bacterial growth at 25
195 mg/ml. Kaolinite, which has been shown to preserve experimentally buried invertebrate
196 carcasses better than quartz, calcite, and montmorillonite, also inhibits the growth of
197 sulfate-reducing bacteria, autotrophic methanogens, and a heterotrophic soil bacterium
198 (Wong et al., 2004; Wu et al., 2013; Wilson and Butterfield, 2014; Liu et al., 2016).
199 Natural ‘blue’ clays, which release both Al^{3+} and Fe^{2+} from illite-smectite into solution,
200 are effective antibiotic agents (Morrison et al., 2016); these two ions work synergistically
201 to disrupt and oxidatively damage bacterial cells.

202 Our results show that clays impede the growth of decay bacteria, providing clear
203 evidence that they are likely to have played a role in promoting organic preservation
204 (Butterfield, 1990; Petrovich, 2001; Wilson and Butterfield, 2014). However, clay–
205 bacterial interactions are highly specific with respect to both clay mineral composition
206 and bacterial strain. Na-montmorillonite, for example, inhibits sulfate reducers but
207 increases the longevity, growth rate and metabolic activity of several other groups of
208 decomposers, probably due to its tendency to adsorb trace metals from the environment
209 (Kunc & Stotzky, 1974; Hwang and Tate, 1997; Wong et al., 2004; Wu et al., 2013). The

210 chemical changes associated with decomposition are likely to affect the leaching and
211 adsorption behavior of the clay as well as the speciation and toxicity of leached ions
212 (Guida et al., 1991; Andrews et al., 2003; Morrison et al., 2014). Further experiments
213 involving a wider range of relevant strains, mineral species, and environmental
214 conditions are required to unravel the role of clay–microbe interactions in exceptional
215 preservation.

216

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218

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226

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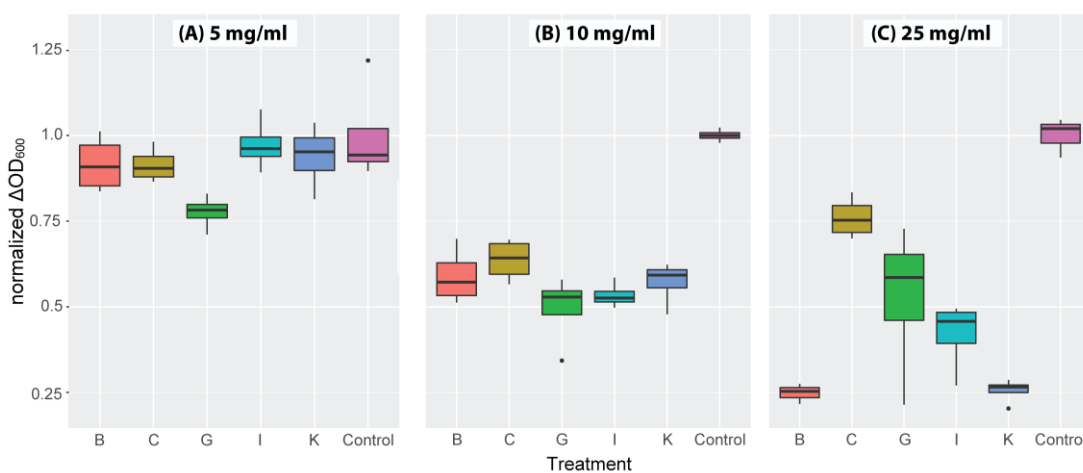
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419 **FIGURE CAPTIONS**

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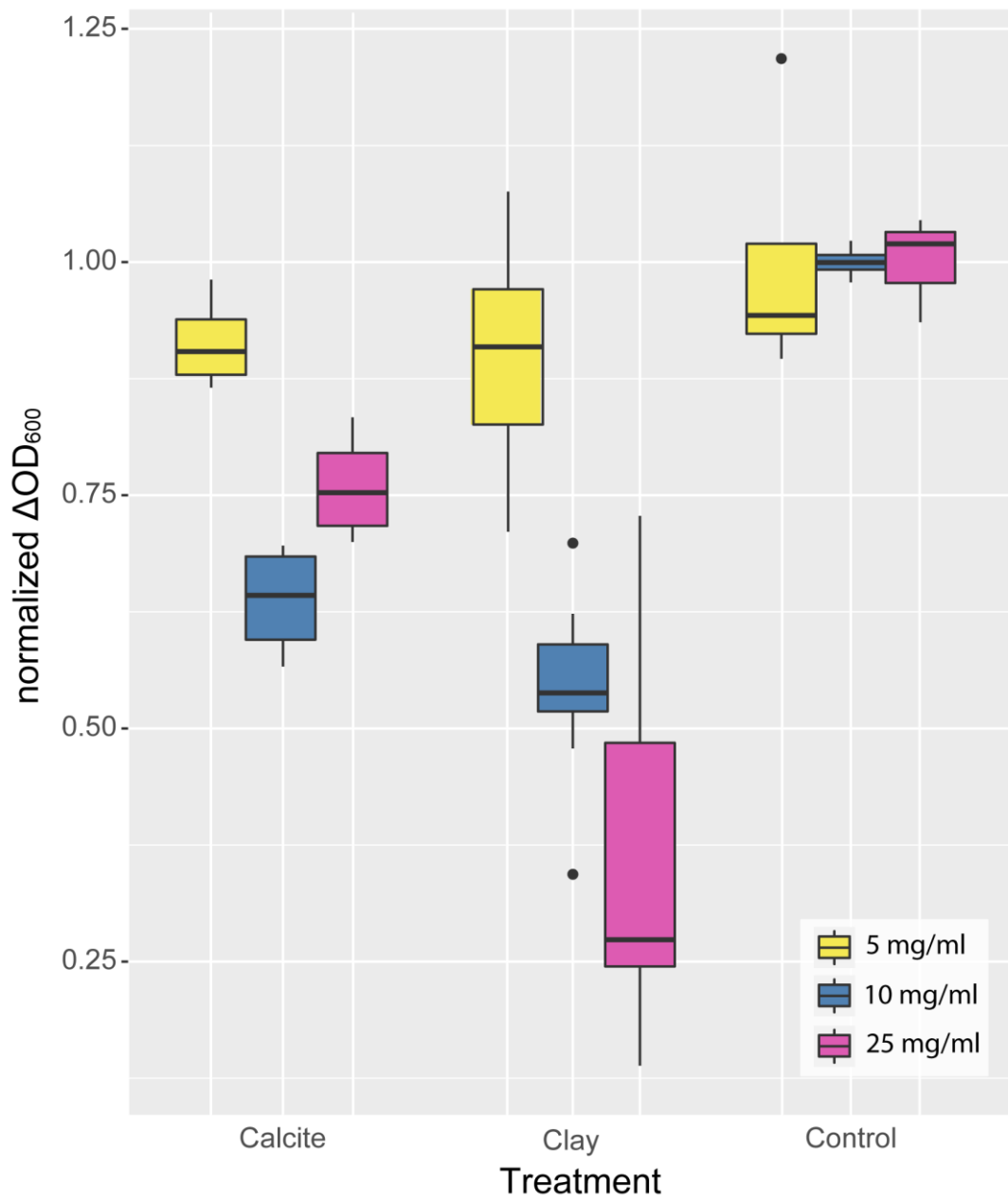
421 **Figure 1.** Normalized optical density at 600 nm (ΔOD_{600}) of six-hour-old
422 *Pseudoalteromonas luteoviolacea* subcultures taken after 24 hours growth with A: 5, B:
423 10 and C: 25 mg/ml <2-3 μ m mineral particles (B = berthierine, C = calcite, G =
424 glauconite, I = illite, K = kaolinite) and with no mineral particles (Control). Floating
425 points are outliers. Experiments were conducted in quadruplicate. Values shown
426 represent increases above $t=0$ and are normalized to the control.



427

428 **Figure 2** Normalized optical density at 600 nm (ΔOD_{600}) of six-hour-old
429 *Pseudoalteromonas luteoviolacea* subcultures taken after 24 hours growth with 5, 10 and
430 25 mg/ml <2-3 μ m mineral particles and with no mineral particles (Control). Berthierine,
431 glauconite, illite and kaolinite are grouped together as Clay. Experiments were conducted

432 in quadruplicate. Values shown represent increases above t=0 and are normalised to the
433 control.



434