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1 **Determination of poly- β -hydroxyalkanoate in Peat**

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9
10 **ABSTRACT**

11 Poly- β -hydroxyalkanoate (PHA) is a prokaryotic energy reserve material which has been used as
12 an indicator of environmental stress in aquatic bacteria. The following technique has been used to
13 quantify PHA in peatland microorganisms. Peat samples were dried, digested in sulphuric acid to
14 convert PHA into crotonic acid, and the resulting acid determined using organic acid high
15 performance liquid chromatography (HPLC) and ultraviolet (UV) detection at 214 nm. This
16 technique is suggested to have potential value as an indicator of environmental stress on peatland
17 microorganisms, such as that caused by summer drought, or changes in soil nutrient availability,
18 which are predicted consequences of climatic change.

19 **KEYWORDS:** High performance liquid chromatography; Microbes; Peatland; Poly- β -
20 hydroxyalkanoate.

22 INTRODUCTION

23 Poly- β -hydroxyalkanoate (PHA) is an energy reserve polymer found in almost all genera of the
24 bacterial kingdom and can accumulate to levels as high as 90% of the cell dry weight (Madison
25 and Huisman 1999; Kadouri et al. 2005). The ability to produce PHA is an adaptation to
26 environments where nutrients are scarce or exhibit temporal fluctuations (Koller et al. 2011). Many
27 bacteria deposit energy reserve material when their growth is restricted in the presence of excess
28 carbon and/or an energy source, which is then utilized when the energy supply from exogenous
29 sources is no longer sufficient for optimal maintenance of the cell (Dawes 1976). For this reason,
30 PHA has been used as an indicator of changes in nutrient availability, and particularly nutritional
31 stress, on microorganisms. PHA can be used as an indicator of nutritional stress because: (i)
32 increasing the carbon (C): nitrogen (N) ratio of microbial growth media increases the PHA content
33 of certain bacteria (Macrae and Wilkinson 1958), and (ii) certain bacteria metabolize their PHA
34 store when starved of energy and nutrients (Malmcrona-Friberg et al. 1986). Furthermore,
35 estuarine bacteria can accumulate PHA following disturbance to the redox potential of the
36 sediment (Herron et al. 1978), whereas biofilms in rivers can use up their PHA reserves following
37 a change in environmental conditions until the biofilm acclimates to the new conditions (Freeman
38 et al. 1993a).

39 In soils, changes in temperature or water availability can result in changes to nutrient availability
40 and soil redox potential, which may place bacteria under stress. Because water is the most
41 important factor regulating the biogeochemistry of wetland soils (Fenner et al. 2009), and the
42 frequency of severe droughts in upland UK is reported to be increasing (Worrall et al. 2006), this
43 effect is currently particularly important for study. Although measurement of PHA has been
44 undertaken in a saline wetland substrate (Ramezani et al. 2014), a number of wetland analogues,

45 such as intertidal and marine sediments (Findlay and White 1983; Herron et al. 1978) as well as in
46 rivers (Freeman et al. 1993a; Freeman et al. 1994; Lòpez et al. 1995) and lakes (van Gernerden et
47 al. 1985; Mas-Castellà and Guerrero 1995), as yet, PHA has not been measured in peat. This paper
48 presents the results of a preliminary experiment aimed at adapting a technique used to determine
49 the PHA content of laboratory cultures of bacteria (Karr et al. 1983) for the determination of the
50 PHA content of microorganisms in peat soils. The technique, which involves the digestion of peat
51 samples in sulphuric acid to convert PHA into crotonic acid, which is quantified using organic acid
52 high performance liquid chromatography (HPLC) and ultraviolet (UV) detection at 214 nm, was
53 used on peat samples from a minerotrophic fen.

54

55 **MATERIALS AND METHODS**

56 **Collection of Samples**

57 Peat was collected from a minerotrophic fen at Cors Goch, on Anglesey, Wales, UK (NGR SH
58 496813). The site is an M13a mire, dominated by *Festuca rubra* and *Juncus acutiflorus*. The pH
59 of the pore water 5 cm below the peat surface was 5.4. The top layer of vegetation was removed
60 and peat collected from the top 5 cm of the profile. This peat was used for method development
61 purposes. Subsequently, the method was also tested on samples of drained and pristine peat,
62 collected using the same procedure, from an area of tropical peat swamp near Bukit Kemuning,
63 Selangor, Malaysia, approximately 80 km northwest of Kuala Lumpur. The pristine site was
64 dominated by peat forest trees, such as *Koompassia malaccensis*, *Shorea uliginosa*, and *Santiria*
65 spp. The drained site has been used principally for mixed agriculture, including the growing of
66 pineapples, following drainage approximately 10 years previously.

67 **PHA Analysis**

68 Samples were analysed using a modification of the method of Karr et al. (1983), whereby PHA is
69 converted to crotonic acid by sulphuric acid digestion at 90°C. A heating block enabled samples
70 to be digested in batches. During preliminary studies, samples of the fen peat were either digested
71 wet or oven-dried at 60°C to determine whether water reduces the efficiency of the sulphuric acid
72 digestion. Peat samples (0.01 g) were digested in 1, 2, 3, 4, and 5 mL of sulphuric acid and over a
73 range of digestion times up to 2 hours to determine the optimal digestion conditions at 90°C. After
74 cooling the digested samples for 30 minutes, samples were diluted 1:20 with deionized water and
75 analysed by HPLC, based on the method of Karr et al. (1983), also used by Freeman et al. (1993a).
76 A DIONEX AI-450 (Dionex Corp., Sunnyvale, CA) chromatograph with a VDM-II variable
77 wavelength detector set at 214 nm was fitted with an Aminex HPX-87H organic acid HPLC
78 column (Bio-Rad, Hemel Hempstead, UK). Sulphuric acid (0.028 M) was used as the eluent at a
79 flow rate of 0.7 mL min⁻¹. Crotonic acid had a retention time of 26.5 minutes. Using this detector,
80 the crotonic acid standard was linear up to at least 50 mg L⁻¹ ($r^2 = 1.000$).

81 Statistically significant differences in the conversion of PHA to crotonic acid were investigated
82 using ANOVA on Minitab, version 9.2 (Minitab Inc., Coventry, UK).

83

84 **RESULTS AND DISCUSSION**

85 Using a digestion volume of 1 mL sulphuric acid, significantly more PHA was converted to
86 crotonic acid using dried peat samples compared with waterlogged peat samples (Figure 1;
87 ANOVA; $p < 0.001$). However, for all other sulphuric acid volumes no difference was observed
88 between the dried and waterlogged samples. In the waterlogged peat samples, maximum

89 conversion of PHA to crotonic acid was observed using 4 mL of sulphuric acid. No more PHA
90 was converted to crotonic acid using 5 mL of digestion mixture. In the dried peat samples,
91 maximum conversion of PHA to crotonic acid was observed using 3 mL of sulphuric acid. Though
92 there was no significant difference between using dry peat with 3 mL of sulphuric acid, or wet peat
93 with 4 mL of sulphuric acid, it is preferable to use dried peat with less acid, because larger volumes
94 of acid entail a greater sample dilution with water, decreasing the HPLC detection limit. Using 4
95 mL of sulphuric acid and waterlogged peat, 30 minutes was demonstrated to be the optimal
96 digestion time (Figure 2). After 30 minutes, conversion of PHA to crotonic acid fell slightly before
97 appearing to plateau.

98 Using the optimum conditions established for measuring PHA concentration in waterlogged
99 samples (digestion in 4 mL of sulphuric acid for 30 minutes), PHA measurement of a Malaysian
100 peat was undertaken. The method was sufficiently sensitive to identify a significant difference
101 between the pristine ($1.76 \pm 0.40 \text{ mg L}^{-1} \text{ crotonic acid g}^{-1} \text{ peat}$) and drained site ($2.38 \pm 0.18 \text{ mg}$
102 $\text{L}^{-1} \text{ crotonic acid g}^{-1} \text{ peat}$). These values were both substantially higher than the equivalent
103 measurement for the fen peat ($0.62 \pm 0.06 \text{ mg L}^{-1} \text{ crotonic acid g}^{-1} \text{ peat}$) and may be related to
104 higher productivity in the tropical peat.

105 The status of wetland systems as carbon sinks is dependent on the prevalence of waterlogged
106 (anaerobic) conditions, since this acts to suppress the microbial degradation of organic material
107 (Freeman et al. 2001). Increased drought in wetlands, a predicted consequence of climatic change
108 (Worrall et al. 2006), may result in lowered water table levels, which can widen the aerobic zone
109 at the surface of the peat and result in significant changes to, for example, the rate of nutrient
110 release from the peat (e.g. Heathwaite 1990; Freeman et al. 1993b). Nutrient availability may
111 determine the rate of other biogeochemical processes in soils. For example, nitrate release

112 following water table drawdown (Freeman et al. 1993b) can result in significant increases in
113 nitrous oxide production from peatlands by microbial denitrification (Dowrick 1998). Since PHA
114 can act as an indicator of nutritional stress in microbial communities (Koller et al. 2011), it could
115 offer a useful means of assessing the impact of climate change on the microbial response of
116 peatlands, which may have important consequences for the stability of peatland carbon stocks and
117 greenhouse gas emissions.

118

119 **CONCLUSIONS**

120 The present study demonstrates for the first time, the potential for direct measurement of PHA
121 concentrations in peat samples. We recommend further validation work using the method
122 presented here including measurement of PHA concentrations over a wider range of experimental
123 conditions, at different substrate depths and in additional peatland environments. Measurements
124 of PHA over a range of nutritional conditions could also help to assess the value of PHA as an
125 indicator of nutritional stress in peatland environments.

126

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