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Long-term survival of bryophytes underground: an investigation of the diaspore bank of *Physcomitrium eurystomum* Sendtn.

Des A. Callaghan [a], Heinjo During[b], Rafael Medina [c] and Handong Yang[d]

[a]Bryophyte Surveys Ltd, Almondsbury, South Gloucestershire BS32 4DU, UK; [b]Utrecht University, Utrecht, The Netherlands; [c]Complutense University of Madrid, Madrid, Spain; [d]Environmental Change Research Centre, University College London, London WC1E 6BT, UK

ABSTRACT

Introduction. Bryophyte diaspore banks are a critical aspect of the life strategy of some species yet remain neglected and poorly understood. This study investigated the longevity in natural habitat of the diaspore bank of *Physcomitrium eurystomum* Sendtn., a moss species that is threatened with extinction in Europe.

Methods. Undisturbed soil cores of 40 cm depth were collected from Langmere, Norfolk, UK, and were split into investigated sediment layers of 1 cm depth. Dating of sediment layers was done by an analysis of radionuclides, and diaspore germination trials of each layer were carried out in a growth chamber. DNA barcoding was used to help identify plants that germinated.

Key results and conclusions. Viable diaspores of *Physcomitrium eurystomum* frequently occurred in sediment layers that were at least 100 years old and continued to occur in much lower layers that were probably several hundred years old. The long-term survival of bryophytes underground can have important implications for conservation decisions.

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KEYWORDS
DNA barcoding; Funariaceae; germination trial; lead-210; sediment core; spore longevity

Introduction

Diaspore banks allow many species of bryophyte to survive in a dormant state below ground and may be composed of spores, asexual propagules or fragments of the gametophyte (During 1997, 2001). Investigations involving germination trials have shown that a broad range of habitats around the world support a diverse reservoir of viable diaspores of bryophytes in their soils, for example including Antarctic fell fields, boreal forest, chalk grassland, coastal meadows, cultivated fields, dry tropical savanna, hot deserts, Mediterranean shrubland and woodland, tropical rain forest and various types of freshwater wetlands (Furness and Hall 1981; During and ter Horst 1983; During et al. 1987; Leck and Simpson 1987; Smith 1987; Jonsson 1993; Poschlod 1993; Bisang 1996; Rydgren and Hestmark 1997; Bisang et al. 2003; Eckstein 2006; During 2007; Caners et al. 2009; Smith 2013; Ingerpuu and Sarv 2015; Malkowsky et al. 2018; Callaghan et al. 2020a). In fact, diaspore banks seem to be ubiquitous in soils wherever bryophytes occur and can be a critical aspect of the life strategy of some species. Evidence also suggests that they can have an important role in accumulating genetic variability and may contain haplotypes that are not present in surface populations (Hock et al. 2008).

Physcomitrium eurystomum Sendtn. is an episodic species, typically occupying the exposed mud of freshwater wetlands (Nieuwkoop 2016; Rimac et al. 2019; Callaghan et al. 2020b). It produces sporophytes abundantly and releases its large spores (30–40 µm diameter) close to the soil surface (2–5 mm height). Its germination from soil samples collected from wetland habitats suggests that it forms a persistent diaspore bank (Poschlod 1993; Eckstein 2006; Malkowsky et al. 2018), although the potential longevity of the diaspores has not been investigated. The species has a broad geographical range, including parts of Africa, Asia, Australasia and Europe. Although it is widespread in Europe, it is generally rare and is undergoing a decline; it is included as 'Vulnerable' on the IUCN Red List of bryophytes in Europe (Hodgetts et al. 2019). *Physcomitrium eurystomum* is also rare and undergoing a decline in Britain and is categorised as 'Endangered' on the IUCN Red List of bryophytes in Britain (Callaghan Forthcoming 2023), being confined to Southeast England, where only five sites were found to be occupied during a recent national survey (Callaghan et al. 2020b). The aim of this study is to investigate the diaspore bank of *P. eurystomum*, particularly its potential longevity in natural habitat.

Materials and methods

Study site

About 10 miles north of Norwich, Langmere (52° 27′40′′N, 0°48′19′′E), Norfolk, UK, comprises a natural freshwater wetland of three small basins, with water supplied to them by direct rainfall but mainly from a

CONTACT Des Callaghan des.callaghan@outlook.com

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groundwater aquifer located in the underlying chalk. No watercourses flow into or out of any of the basins. In response to seasonal changes in the chalk aquifer, the water level of each basin fluctuates substantially, and each may remain full or completely dry for long periods. The basins support a number of rare plants typical of exposed mud, including *Physcomitrium eurystomum*. The moss was discovered here, new for Britain, in 1961 by B. F. T. Ducker (Ducker and Warburg 1961). The site is owned and managed as a nature reserve by Norfolk Wildlife Trust and became legally protected for its nature conservation in interest in 1954, via its inclusion within the East Wretham Heath Site of Special Scientific Interest.

Sampling of sediment layers

In October 2019, when all three basins of Langmere were dry, three undisturbed soil cores, each 10 cm apart, were extracted from the centre of the main basin (Figure 1). An Eijkelkamp 04.17.01.C split tube sampler (Eijkelkamp, Giesbeek, The Netherlands) was used to collect the cores; it is designed to provide an undisturbed soil core of 5 cm diameter and up to 40 cm depth, encased during the extraction process within a detachable clear plastic tube that allows easy transport of the final sample (Figure 2). Each core ranged from the soil surface to a depth of 40 cm, throughout which the soil comprised a compacted sandy loam (Figure 3). At the time of extraction from the ground, the water table was substantially below 40 cm depth and the cores were dry throughout their length.

On the following day, each core was split into 40 soil samples, each sample comprising a 1 cm depth sediment layer of 19.6 cm^3. From one of the cores, 10 cm^3 of soil was extracted from each sample and was sent to University College London (UCL) for dating (see below). All remaining samples were sent to the University of Utrecht for a germination trial (see below).

Dating of sediment layers

Dating of sediment layers was carried out at the Environmental Radiometric Facility at UCL by an analysis of radionuclides, including americium-241 (^{241}Am; half-life = 470 years), cesium-137 (^{137}Cs; half-life = 30 years), lead-210 (^{210}Pb; half-life = 22.3 years) and radium-226 (^{226}Ra; half-life = 1600 years). Analysis was by direct gamma assay, using an ORTEC HPGe GWL series well-type coaxial low background intrinsic germanium detector (ORTEC, Easley, SC, USA). Lead-210 was determined via its gamma emissions at 46.5keV, and ^{226}Ra by the 295keV and 352keV gamma rays emitted by its daughter isotope ^{214}Pb following 3 weeks of storage in sealed containers to allow radioactive equilibration. Cesium-137 and ^{241}Am were measured by their emissions at 662keV and 59.5keV, respectively (Appleby et al. 1986). The absolute efficiencies of the detector were determined using calibrated sources and sediment samples of known activity. Corrections were made for the effect of self-absorption of low-energy gamma rays within the sample (Appleby et al. 1992).

Germination trial of sediment layers

Diaspore germination trials were carried out in a growth chamber at Utrecht University for a period of 149 days (29 October 2019 to 26 March 2020). Each

Figure 1. Location at Langmere, Norfolk, UK, from where soil cores were extracted (9 October 2019). Photograph: D. A. Callaghan.

Figure 2. Extraction of undisturbed soil cores at Langmere, Norfolk, UK: (A) the Eijkelkamp 04.17.01.C split tube sampler, and (B) an extracted soil core encased within plastic tubing (9 October 2019). Photograph: D. A. Callaghan.

Figure 3. Soil inspection pit at Langmere, Norfolk, UK, dug after the soil cores had been extracted, to illustrate the soil profile, comprising a compacted sandy loam throughout the 40 cm sample depth (9 October 2019). Photograph: D. A. Callaghan.

soil sample ($n = 120$) was spread over sterilised sand in a separate 8 cm × 8 cm transparent plastic box with a lid. Light was provided at 200 µmol m^{-2} s^{-1} (16 h light, 8 h darkness) and temperature was kept at 20–22°C.

At the end of the trial, boxes that contained *Physcomitrium* were transferred to a window sill in the home of the second author (H.D.) and were monitored for the production of sporophytes for a further 510 days (until 18 August 2021), to help confirm the identification of plants. During the trial, vascular plants were removed from containers as they arose, to avoid competition, and the soil was sprayed with demineralised water, as necessary, to maintain a moist soil surface.

DNA barcoding

Physcomitrium eurystomum can be identified with certainty only when mature sporophytes are present. To help confirm the identity of plants that did not produce sporophytes during the growth trial, DNA barcoding of samples was carried out on a selection of 15 cultures initiated from core sediment. Two negative controls of sterile *P. pyriforme* (Hedw.) Brid. plants, plus two positive controls of *P. eurystomum*, were also barcoded for comparison.

Physcomitrium eurystomum is an allopolyploid species (McDaniel et al. 2010; Ostendorf et al. 2021) resulting from the hybridisation of two different lineages within the genus. This hybrid origin results in the simultaneous presence of slightly different paralogous copies of nuclear loci that can be interpreted from the presence of double peaks in a Sanger sequencing chromatogram.

DNA was extracted using the Norgen Plant/Fungi DNA Isolation Kit (Biotek Corporation, Thorold, Canada) following the instructions provided by the manufacturer. Considering the hybrid origin of the species, two nuclear loci were targeted by PCR. Loci 4780 and 7379 from the set of baits of Medina et al. (2019) were chosen as barcodes based on their usefulness for *Physcomitrium eurystomum* barcoding, demonstrated by Callaghan et al. (2020b) using the set of primers designed by Matthew Johnson and Nikisha Patel: 4780F = ATGGACGGCGCACTTGTTA; 4780R = CTTGTAACGTCGCTTCAGATTTT; 7379F = TCACGTTGGACCATGTGACG; and 7379R = CGTTCAAACGCCTCTCATTG.

Polymerase chain reactions were conducted in final volumes of 50 µL, with 0.15 µL of NZYTaq II DNA polymerase (NZYTech, Lisbon, Portugal), 1 µL of 10 µM dNTP mix, 1 µL of each primer (10 µM) and 1 µL of DNA extract. The thermal cycler was programmed with a hot start denaturation step of 5 min at 94°C, followed by 30 cycles of denaturation (1 min, 94°C), annealing (1 min, 50°C) and extension (1 min, 70°C), ended by a final extension step of 10 min. Amplification products were visualised in 1% agarose gels, cleaned used the ExoSAP-IT protocol (Thermofisher, Waltham, MA, USA), and sent for sequencing to Macrogen through the EZ-sequencing service. Contigs were generated using Geneious (Kearse et al. 2012; http://www.geneious.com), keeping track of all double peaks shown in both reads of each sequence of the nuclear loci using the standard IUPAC ambiguous nucleotide letters in the consensus sequence, and

then compared visually on PhyDe (Müller et al. 2006; http://www.phyde.de).

Results

Dating of sediment layers

Total ^{210}Pb activity reaches equilibrium depth with supported ^{210}Pb activity at ca 12 cm depth (Table 1; Figure 4a). Unsupported ^{210}Pb activities, calculated by subtracting ^{226}Ra activity (as supported ^{210}Pb) from total ^{210}Pb activity, decline more or less exponentially with depth (see Table 1; Figure 4b). Maximum values are close to the surface, suggesting an increase in sedimentation rates in recent years.

The activity of ^{137}Cs shows a peak between 3.5 and 6.5 cm and a higher peak between 1.5 and 2.5 cm (Table 2; Figure 4c). These peaks might be attributable to fallout from the atmospheric testing of nuclear weapons, which reached its maximum level in 1963, and the Chernobyl nuclear accident in 1986, respectively. Low ^{241}Am activities were detected in disconnected samples, which are not sufficient for dating (see Table 2).

Use of the CIC (constant initial concentration) model was precluded by the non-monotonic variation in unsupported ^{210}Pb activities. The ^{210}Pb chronologies were calculated using the CRS (constant rate of ^{210}Pb supply) dating model (Appleby 2001; Table 3). The CRS model places 1963 and 1986 between 3.5 and 4.5 cm and between 1.5 and 2.5 cm, respectively, suggesting that Langmere was affected by the 1986 fallout from the Chernobyl accident. The impact of fallout from the atmospheric testing of nuclear weapons was not obvious. The CRS dating model suggests that since the 1890s, sedimentation rates increased significantly during the 1930s and 1950s but have otherwise remained relatively stable (see Table 1; Figure 5).

Table 1. Concentrations of ^{210}Pb in sediment core taken in October 2019 from Langmere, Norfolk, UK.[a]

Depth (cm)	Dry mass (g cm^{-2})	Total ^{210}Pb (Bq kg^{-1}) (SE)	Supported ^{210}Pb (Bq kg^{-1}) (SE)	Unsupported ^{210}Pb (Bq kg^{-1}) (SE)	Cumulative unsupported ^{210}Pb (Bq kg^{-1}) (SE)[b]
0.5	0.489	177 (15.6)	23.4 (3.85)	154 (16.1)	880 (71.2)
1.5	1.46	193 (19.6)	28.4 (4.76)	165 (20.1)	2430 (182)
2.5	2.50	116 (15.2)	28.1 (3.97)	87.4 (15.7)	3700 (275)
3.5	3.73	57.5 (5.92)	36.5 (1.82)	20.9 (6.19)	4270 (321)
4.5	5.04	40.9 (6.46)	31.0 (1.98)	9.88 (6.76)	4460 (331)
5.5	6.34	38.4 (4.76)	36.9 (1.53)	1.46 (5.00)	4520 (341)
6.5	7.62	51.8 (6.42)	36.5 (1.98)	15.3 (6.72)	4600 (349)
7.5	8.92	51.6 (5.85)	37.8 (1.82)	13.8 (6.13)	4790 (359)
8.5	10.2	40.2 (5.04)	34.1 (1.54)	6.18 (5.27)	4910 (367)
9.5	11.5	46.0 (6.23)	31.1 (1.55)	14.9 (6.42)	5040 (374)
10.5	12.8	39.6 (4.75)	27.4 (1.44)	12.2 (4.96)	5210 (383)
11.5	14.0	46.8 (5.41)	31.2 (1.46)	15.6 (5.60)	5380 (388)
12.5	15.3	18.9 (9.37)	29.3 (1.41)	−10.4 (9.48)	–

SE, standard error.
[a]Values are shown to three significant figures.
[b]No value is given for cumulative unsupported ^{210}Pb at 12.5 depth, because at that point unsupported ^{210}Pb activity tends to zero.

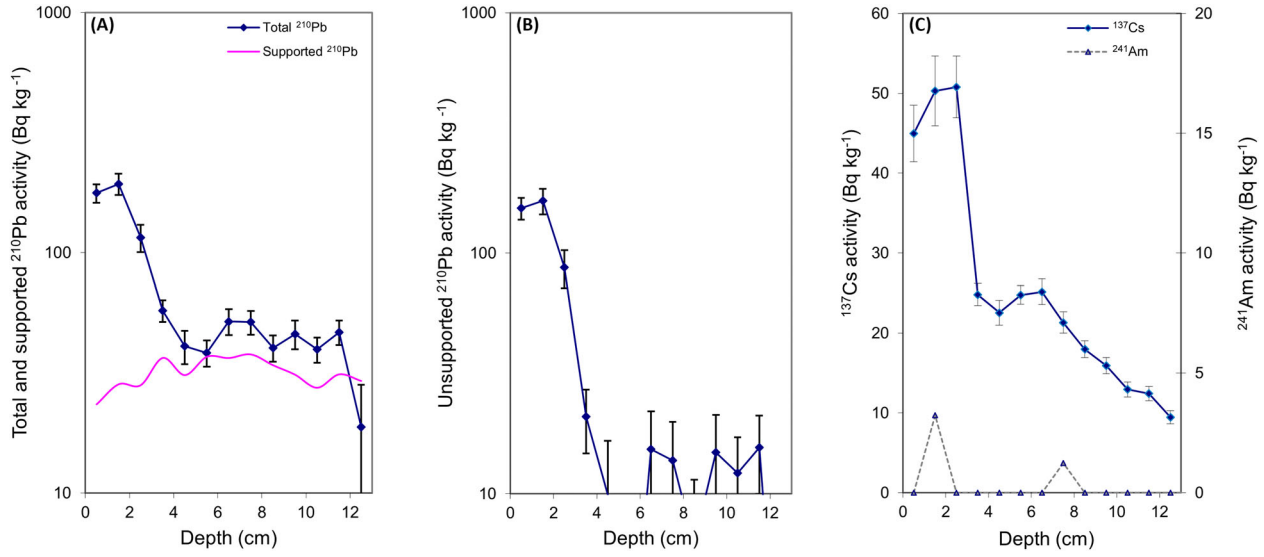

Figure 4. Fallout radionuclide concentrations in sediment core taken from Langmere, Norfolk, UK, showing (A) total and supported ^{210}Pb, (B) unsupported ^{210}Pb, and (C) ^{137}Cs and ^{241}Am concentrations versus depth.

Table 2. Artificial fallout radionuclide concentrations in sediment core taken in October 2019 from Langmere, Norfolk, UK.[a]

Depth (cm)	^{137}Cs (Bq kg^{-1}) (SE)	^{241}Am (Bq kg^{-1}) (SE)
0.5	45.0 (3.56)	0
1.5	50.3 (4.37)	3.24 (1.64)
2.5	50.8 (3.86)	0
3.5	24.8 (1.39)	0
4.5	22.6 (1.54)	0
5.5	24.8 (1.15)	0
6.5	25.2 (1.59)	0
7.5	21.3 (1.32)	1.24 (0.61)
8.5	18.0 (1.05)	0
9.5	15.9 (1.01)	0
10.5	12.9 (0.93)	0
11.5	12.4 (0.90)	0
12.5	9.45 (0.83)	0

SE, standard error.
[a]Values are shown to three significant figures.

Table 3. Chronology of ^{210}Pb in sediment core taken in October 2019 from Langmere, Norfolk, UK.[a]

		Chronology		Sedimentation rate		
Depth (cm)	Dry mass (g cm^{-2})	Date (AD)	Age (years) (SE)	g cm^{-2} year^{-1}	cm year^{-1}	± %
0.5	0.489	2013	6 (3)	0.090	0.093	10.3
1.5	1.46	1999	20 (3)	0.0548	0.054	11.8
2.5	2.50	1981	38 (4)	0.0581	0.051	23.5
3.5	3.73	1967	52 (6)	0.158	0.125	36.2
4.5	5.04	1961	58 (7)	0.274	0.21	72.1
5.5	6.34	1957	62 (7)	0.356	0.276	93.3
6.5	7.62	1955	64 (8)	0.150	0.116	49.7
7.5	8.92	1946	73 (9)	0.124	0.095	52.7
8.5	10.2	1938	81 (11)	0.213	0.165	91.1
9.5	11.5	1926	93 (13)	0.0621	0.048	58.3
10.5	12.8	1898	121 (26)	0.0316	0.025	64.6

SE, standard error.
[a]Values are shown to three significant figures.

DNA barcoding

All but one of the attempted amplifications (7379 locus of sample B090) were successfully sequenced. We used the sequences obtained in Callaghan et al. (2020b) for loci 4780 and 7379 (e.g. GenBank accessions MT158336 and MT158340) as references to compare the newly obtained sequences. All the sequences and double-peak patterns from cultures initiated from the cores and the two positive controls matched completely with the reference sequences for *Physcomitrium eurystomum*. The sequences obtained from the negative controls matched the reference sequences of *P. pyriforme* obtained in Callaghan et al. (2020b), corresponding to MT158338 and MT158342 GenBank accessions.

Germination trial of sediment layers

Germination of *Physcomitrium eurystomum* occurred in many boxes ($n = 61$; 51%), and subsequent production of sporophytes occurred in 23 (38%) of the occupied boxes (Figures 6, 7). The first sporophytes were noted on 4 February 2020, 68 days from the start of the trial. All the *Physcomitrium* plants that produced sporophytes were *P. eurystomum*, and no related species were detected. Figure 7 shows the germination of the species from the different sediment layers of the three soil cores. Results indicate that viable spores of *P. eurystomum* at Langmere are concentrated in the upper 20 cm of soil but also occur frequently lower down, including at the maximum depth sampled (39–40 cm).

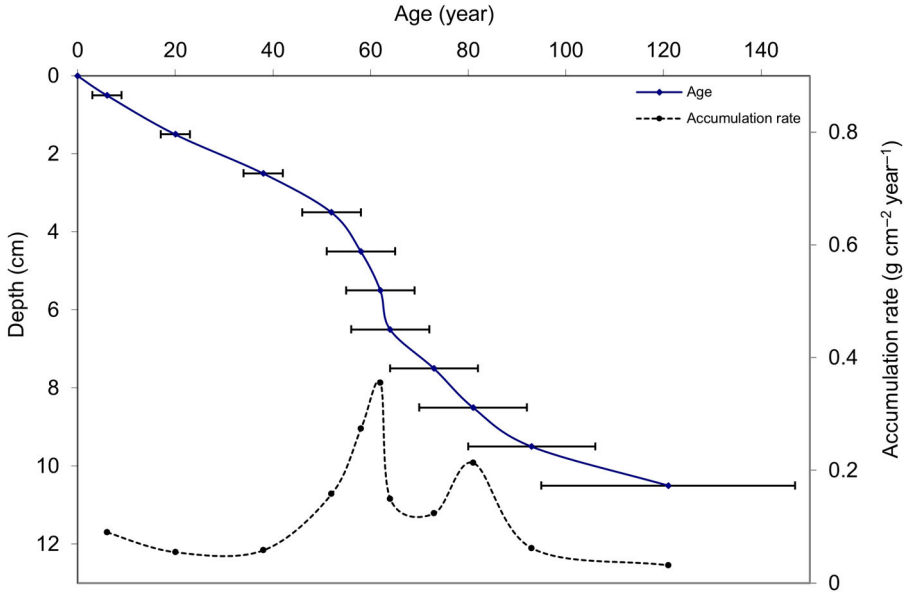

Figure 5. Radiometric chronology of sediment core taken from Langmere, Norfolk, UK, showing the CRS model ^{210}Pb dates and sedimentation rates.

Figure 6. Shoots and sporophytes of *Physcomitrium eurystomum* arising from the germination trial of a soil sample aged 73 (± 9) years. Photograph: H. J. During.

Discussion

This study shows that viable diaspores of *Physcomitrium eurystomum* frequently occur in sediment layers that are at least 100 years old and continue to occur in much lower layers that are probably several hundred years old. Only a few other studies have investigated the potential longevity of bryophyte diaspore banks. Bristol (1916) reported the growth of moss protonema from soil samples aged 45–48 years, collected from arable fields at Rothamsted Experimental Station (UK), which Whitehouse (1984) considered was probably produced by *Dicranella staphylina* H.Whitehouse, and Dyer and Lindsay (1992) documented bryophyte growth from forest soil aged at least 100 years from North Carolina, USA. Duckett and Clymo (1988) reported the frequent regeneration of liverworts from peat layers aged up to 10 years and more infrequently from layers up to about 40 years from a peatland in the New Forest, UK, plus the regeneration of *Sphagnum* up to the maximum depth they aged (70 years). Bu et al. (2017) germinated *Sphagnum* spores from Hani Peatland, China, from peat layers aged up to 680 years, and based on statistical models indicated that some spores may remain viable for over 1000 years. Also in China, Wang et al. (2020) predicted the longevity (i.e. the burial time when 1% spores are viable) of *Sphagnum* spore banks in the Changbai Mountains to be ca 390 and ca 140 years for low- and high-elevation peatlands, respectively. In permanently frozen conditions, regeneration of bryophyte shoots has been confirmed from glacial ice that was 400 years old (La Farge et al. 2013) and from permafrost aged at least 1530 years (Roads et al. 2014). Thus, the results of the present study add to the small but growing body of evidence that the diaspores of some bryophytes have evolved a capacity to remain viable for a surprisingly long time, even in conditions where they are not permanently frozen.

The long-persistent diaspore bank of *Physcomitrium eurystomum* allows individuals to wait in situ for a prolonged period until suitable growth conditions arise, and helps the species to occupy a habitat where such conditions may not occur for many years but eventually re-occur in the same general location (i.e. growth and reproduction episodes are temporally unpredictable but spatially predictable). When suitable conditions occur, this study also shows that the species is capable of rapid growth and reproduction, with the first production of sporophytes noted within 68 days of germination. This helps the species to survive in a habitat where conditions suitable for growth and reproduction occur only briefly, which is reflected in observations of the species in Britain, whereby it can be abundant at sites in favourable years but may otherwise not be seen for long periods (Callaghan et al. 2020b). Similarly, Furness and Hall (1981) found from observations over 44 years that the intermittent appearance of *P. sphaericum* (C.Ludw.) Brid. on the exposed mud of a reservoir margin was correlated with summer droughts. A similar life strategy has been adopted by other exposed mud specialists, such as *Micromitrium tenerum* (Bruch & Schimp.) Crosby (Schmidt and Kohn 1993; Eckstein 2006; Callaghan 2021), *P. patens* (Hedw.) Mitt. (Malkowsky et al. 2018) and *Riccia huebeneriana* Lindenb. (Eckstein 2006).

Conservation assessments of the population status of bryophyte species that occur above ground only briefly are often associated with greater uncertainty because records of their occurrence are often limited, leading to increased measurement error, as illustrated for *Costesia macrocarpa* (Schimp.) Cuvertino, Miserere & Buffa in Chile (Larraín et al. 2019). The possible

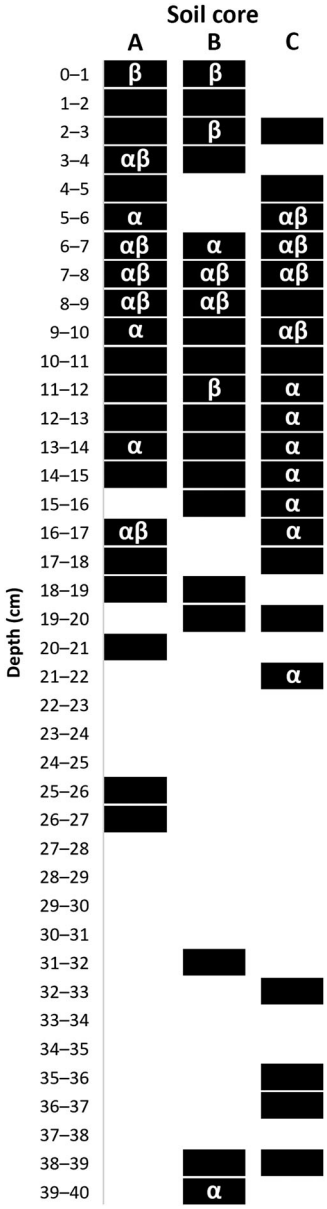

Figure 7. Germination of *Physcomitrium eurystomum* from different sediment layers of three soil cores taken from Langmere, Norfolk, UK. The species identification method used for a sample is indicated by (α) sporophyte characters ($n = 23$) or (β) DNA sequence analysis ($n = 15$). Samples for which neither of these methods were available for identification ($n = 34$) are assumed to be *P. eurystomum* because their gametophyte characters closely matched those of the confirmed samples and possible confusion species were not otherwise found during the germination trial.

occurrence of long-persistent diaspore banks substantially increases the uncertainty of conservation assessments. For example, *Meesia longiseta* Hedw. had been classified as extinct in Estonia but was detected subsequently during a germination trial involving soil samples collected from a fen where it was absent above ground (Ingerpuu and Vellak 2018; Ingerpuu et al. 2018).

Commonly, if a species has not been seen at a site for several decades and recent searches have failed to refind it, it is assumed to have become extinct and no further attention is given to it, other than possible reintroduction. If a long-persistent diaspore bank may be present, a more cautious approach is necessary, because suitably targeted habitat management could lead quickly to population regeneration. An illustrative example is provided by Nováková et al. (2015), who describe the appearance of *Physcomitrium eurystomum* from the diaspore bank of shallow pools, after they had been restored by excavation, in an area of the Moravian Karst, Czech Republic, where the species had not previously been recorded. Similar management could usefully be trialled at sites where *P. eurystomum* has been known to occur but at which its continued survival is of concern due to currently unfavourable habitat conditions, at least at sites in Europe where it is a species of high conservation concern. The results of this study suggest it may often survive at such sites, hidden below ground as dormant spores.

This study helps to confirm that bryophyte diaspore banks can be a critical aspect of the life strategy of some species and that some diaspores may remain viable in natural habitat for a remarkably long time, which can have important implications for conservation decisions. It is hoped this will help to encourage further research into bryophyte diaspore banks, because they remain an aspect of bryophyte ecology that is neglected and poorly understood.

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Disclosure statement

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Notes on contributors

Des Callaghan is a consultant bryologist operating under Bryophyte Surveys Ltd. His research is focused on threatened species, conservation ecology and taxonomy.

Heinjo During is a retired plant ecologist and bryologist of the Ecology and Biodiversity Group of Utrecht University.

Rafael Medina is assistant professor at Complutense University of Madrid. His main research interest is bryophyte and fern systematics.

Handong Yang is a principal research fellow working at University College London. His main research interest is environmental pollution and sediment dating.

ORCID

Des A. Callaghan http://orcid.org/0000-0002-0415-1493
Rafael Medina http://orcid.org/0000-0001-5629-1503

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