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Effects of Sediment Preparation Protocol on Cyanobacterial Microfossil Recovery from Lake Sediments

Anjal Mariya

amariya09@qub.ac.uk

Queen's University Belfast

Lynda Bunting

University of Regina

Peter R. Leavitt

University of Regina

Helen M. Roe

Queen's University Belfast

Research Article

Keywords: Cyanobacterial microfossils, Non-pollen palynomorphs, Akinetes, Shealths, Eutrophication, cyanoHABs

Posted Date: November 24th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11660385/v1

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Additional Declarations: No competing interests reported.

Abstract

Increasing prevalence of harmful cyanobacteria algal blooms in global waters poses a significant threat to aquatic life and to human and animal health. Despite considerable progress in monitoring and detection, many freshwater systems lack long-term records of bloom development. In principle, paleolimnological studies based on sediment proxies, including biochemical indicators (e.g., fossil pigments, eDNA) and microfossils (akinetes, heterocytes, sheaths), are a valuable tool for investigating past cyanobacterial bloom dynamics, nutrient fluctuations, and water quality changes. However, relatively little is known of how sediment preparation techniques may affect estimation of historical population densities. Here we use a two-by-two factorial experiment to evaluate the unique and interactive effects of sediment sieving and acetolysis on the recovery of cyanobacterial microfossils from surface (0–1 cm) sediments of two eutrophic lakes (Lough Aghery, Ballysaggart Lough) in Northern Ireland, UK. Each of four treatments were run in triplicate and included a common mild preliminary treatment with KOH to disperse sediments, an addition of *Lycopodium clavatum* spores to standardise counts, and a brief (5 sec) sonication to reduce clumping of akinetes. Results show substantial variation in microfossil abundance across treatments, with consistently and significantly higher microfossil recovery in the least intensive treatment (-sieve, -acetolysis), while acetolysis reduced akinete concentrations 85–95% and biased taxonomic representations due to selective loss of *Aphanizomenon* spp. akinetes relative to those from *Anabaena* / *Dolichospermum* spp. or sheaths from *Gloeotrichia* spp. Together, analyses suggest that minimal processing (+ KOH, +sonication, -sieve, -acetolysis) produces the least-biased recovery of cyanobacterial remains, at least for these genera and similar sedimentary environments.

Introduction

The frequency and magnitude of cyanobacterial harmful algal blooms (CyanoHABs) are rising in global inland waters (Huisman et al. 2018) with elevated biomass or toxin production degrading lacustrine ecosystem functions, fisheries, aquaculture, real-estate values, drinking water supplies, and human or animal health (Anderson 2009; Huisman et al. 2018; Igwaran et al. 2024; Schaeffer et al. 2024). Eutrophication, climate warming, stratification, altered precipitation patterns, and hydrologic changes are the main causes of blooms, with interactions among forcing agents acting to intensify bloom development (Byl et al. 2025; Graham et al. 2016; Paerl and Huisman 2009; Song et al. 2023). Despite advances in monitoring and observational techniques to better understand the modern bloom dynamics (e.g., *in situ* sondes, remote sensing, etc.), most lakes lack long-term records of bloom occurrence, making it challenging to identify cyanobacterial outbreaks, measure processes behind the ecosystem changes, or minimise their impacts (Barouillet et al. 2023). In principle, analysis of cyanobacterial fossils in lake sediments can be used to quantify bloom onset, developmental trajectory, maximum magnitude, and damages associated with cyanobacterial occurrence (e.g., Bunting et al. 2007, 2016, Gushulak et al. 2024).

Palaeolimnological analyses offer a wide array of proxies to estimate past abundance of cyanobacteria, including fossil pigments (carotenoids), sedimentary nucleic acids (16S ribosomal RNA), toxins (microcystins, anatoxins, etc.), and microfossils (akinetes, heterocytes, filament sheaths), while changes in physical and chemical properties of sediments (e.g., stable isotopes, nutrient concentrations, x-ray diffraction) allow reconstruction of the factors favouring bloom development (Erratt et al. 2026, and references therein). Among these proxies, cyanobacterial microfossils, also known as non-pollen palynomorphs (NPPs), have been quantified by phycologists due to their presence on slides processed for pollen quantification (van Geel et al. 1994). Preserved cyanobacterial cell remnants mainly belong to the orders Chroococcales (non-nitrogen fixing) and Nostocales (nitrogen fixing), and their presence has been used to indicate increased nutrient availability, degraded water quality, altered nitrogen dynamics, and elevated environmental stress in freshwater ecosystems (Bunting et al. 2016; Hillbrand et al. 2014; McCarthy et al. 2021; Monecke et al. 2018; Van Geel et al. 1994). Further, direct comparisons demonstrate that some cyanobacterial microfossil concentrations are linearly correlated to their corresponding phytoplankton densities (Bunting et al. 2007). Despite their ability to indicate historical occurrence of bloom-forming taxa, microfossils are often underused relative to other palaeolimnological proxies, possibly because of their microscopic size and the fact that they may be visually obscured by amorphous organic matter (McCarthy et al. 2021). These issues are often addressed by physical (sieving) or chemical (digestion) processing of sediments, despite limited understanding of the consequences of such manipulations on the accuracy of paleolimnological reconstructions.

Although there are several approaches to prepare lake sediments for microscopic analysis of NPPs including cyanobacterial microfossils, the most widely used treatments involve sieving dilute sediments through mesh to remove large particles and acetolysis to digest more labile organic matter (Limaye et al. 2007; McCarthy et al. 2026; Szal et al. 2014; Van Geel 2002). Because many microfossil-producing cyanobacteria are colonial (e.g., *Aphanizomenon, Anabaena/Dolicospermum*, *Gloeotrichia*), use of fine meshes in sediment processing may alter density and composition of recovered remains. Similarly, acetolysis usually involves application of an acetic anhydride - sulphuric acid solution to remove non-sporopollenin components of pollen grains, reduce non-pollen organic remains, and enhance visibility by facilitating swelling of pollen grains (Erdtman 1960; Halbritter et al. 2018; Moore et al. 1991). This treatment is known to impair various NPPs including *Trachelomonas* (Shumilovskikh et al. 2019), *Pediastrum* (Komárek and Jankovská 2001), desmids (Riddick et al. 2017), coprophilous fungi (van Asperen et al. 2016) and dinoflagellate cysts (Mertens et al., 2009). In contrast, there has been limited direct investigations of whether acetolysis degrades cyanobacterial microfossils or alters proportions of preserved taxa (e.g., N_2 fixers vs. non-N_2-fixers).

In principle, acetolysis should affect the recovery of cyanobacterial microfossils from lake sediments. During acetolysis, the mixture of sulphuric acid and acetic anhydride generates acetylium ions (CH_3CO^+) that can cleave the glycosidic bonds by acetylation and ring opening of carbohydrate molecules (Guthrie and McCarthy 1967; Kamerling and Gerwig 2007). This treatment causes breakdown of complex carbohydrate structures into smaller soluble units, contributing to the degradation of polysaccharide-

derived materials (Kamerling and Gerwig 2007). As cyanobacterial cell walls are composed of peptidoglycan (murein) which is a heteropolysaccharide polymer linked by glycosidic bonds, it may also be vulnerable to acetolysis. This observation gives rise to concerns regarding potential bias in palaeolimnological reconstructions based on standard palynological slide preparations, particularly for taxa whose remains appear relatively delicate (e.g., *Aphanizomenon* spp.).

This study is designed to compare four microfossil-isolation protocols ranging from minimal chemical treatment without sieving (-seive) or acetolysis (-acetolysis) to more intensive approaches (+ sieve, +acetolysis) to quantify how microfossil abundance and assemblage composition varies with the degree of sediment treatment. Specifically, we ran a 2x2 factorial design in triplicate with sediments from two eutrophic lakes subject to cyanobacterial blooms to quantify the unique and interactive effects of sieving and acetolysis on estimation of historical cyanobacterial abundance. The aim of this paper is to identify potential methodological biases and enhance the reliability and robustness of cyanobacterial microfossil applications in palaeolimnological studies.

Materials and methods

Study site

Northern Ireland lakes, Lough Aghery and Ballysaggart Lough (Fig. 1), were selected to optimize development of protocols for cyanobacterial microfossil recovery owing to their recent and recurring cyanobacterial blooms (Northern Ireland Environmental Agency, unpublished data). Lough Aghery (54°24'57.2"N, 6°00'53.0"W) is a natural lake situated within the Quiole River catchment in County Down and is presently 2.2 m deep with surface area of 29.5 ha. This eutrophic lake is characterised by relatively high production (Northern Ireland Environmental Agency, unpublished data). The lake is encircled by a drumlin landscape that contain farmland and fringing vegetation, and is used for recreational purposes. Ballysaggart Lough (54°29'40"N 6°46'40"W) is a shallow mill-dammed eutrophic lake in the County Tyrone which is 4.7 m deep with surface area 16 ha. The lake is situated in a suburban area and is surrounded by agricultural lands within the River Blackwater sub-catchment of Lough Neagh.

Experimental design

This study aims to test four methods ranging from minimally processed to intense chemical treatment of sediments to evaluate their effectiveness in recovering cyanobacterial microfossils for microscopic analysis (Fig. 2). The methods were adapted from previous palaeoecological studies, including Van Geel (2002) and Bunting (2005) and contrast the effects of sieving and acetolysis of sediments on cyanobacterial microfossil recovery. Following the addition of exotic *Lycopodium* spores to standardize counting efforts, sediment samples were dispersed in the KOH, subjected to respective sieving and acetolysis treatments and finally a light sonication to avoid microfossil clumping.

Prior to processing, lake sediments were homogenised and subsampled in triplicate to determine the reproducibility and recovery efficiency. Two *Lycopodium clavatum* spore tablets (Batch no: 101023231; Department of Geology, Lund University, Sweden) were dissolved in a 10% HCl solution, centrifuged, washed, and added to each sample to allow standardization of enumeration effort based on projected exotic spore concentration. In addition, after each method of sample preparation, all microfossil concentrates were subject to a 5-second ultrasonic treatment using Cole-Parmer VCX 400 Ultrasonic Homogenizer at 51% amplitude before application of the suspension to slides to reduce the clumping of particles and improve microfossil visibility. Prior testing of sonication duration revealed that a mild treatment (3–5 seconds) had no observable effect on fossil density or composition, although the 5-second trial more effectively dispersed microfossil aggregates. However, more prolonged sonication of > 10 seconds could destroy the more delicate *Aphanizomenon* spp. and *Anabaena* spp. (or *Dolichospermum*; as *Anabaena* hereafter) remains.

Figure 2. Schematic representation of the four sediment preparation methods used for cyanobacterial microfossil analysis. M1 = no sieving and no acetolysis; M2 = sieving only; M3 = acetolysis only; M4 = sieving and acetolysis.

Standardised preparation and counting procedures were followed throughout all treatments to enable methodological comparison. Method 1 (M1) involves the minimal processing of the sediments following Bunting (2005). Fresh sediment samples (2.0 ± 0.5 mL) were added to the measuring cylinder containing a known volume of 10% potassium hydroxide (KOH). Displacement by added KOH was noted for estimating the volume of the sediment taken. The prepared suspension of exotic spores was then combined with the sediment-KOH mixture and an additional aliquot of 10% KOH was added to bring the final sample volume to 40 mL. This solution was kept in water bath of 100°C for ten minutes before settling them for 24 hours for removing humic acid and deflocculating the sediments. Samples were allowed to settle for another 24 hours after washing with distilled water. Later the water was decanted, and glycerol was added in an amount equal to that of the processed sediment.

Method 2 (M2) is identical to M1 with an additional step of sieving with 212-µm pore sieve before washing the sediment-KOH solution with distilled water. This sieve aperture was selected following the established non-pollen palynomorph preparation protocol of Van Geel (2002), which employed 215 µm mesh size. Sieving is intended to remove larger undigested particles which might obscure enumeration of microfossils.

Method 3 (M3) is adapted from Van Geel (2002) by omitting the sieving step. This method is a more chemically intensive than M1 or M2 and is thought to eliminate coloured organic matter that may obscure microfossil visibility. The method follows the same initial steps, allowing washing of the sediment-KOH solution until the supernatant is clear. Later, the remaining particulate suspension was concentrated via centrifugation for 5 min at ~ 3,000 × g (4,000 rpm) then dehydrated with 96% acetic acid. Acetolysis was conducted by adding a mixture of 5 mL containing sulphuric acid and acetic anhydride in 1:9 ratio (by volume) and heating in a water bath at 100°C for five minutes. After cooling, the

sample was centrifuged and washed in distilled water. Later the suspension was washed with alcohol and reconcentrated via centrifugation prior to addition of a known volume of glycerol as above.

Method 4 (M4) follows the same procedure as M3 with the additional step of using the 212-μm aperture sieve after the settling the KOH-sediment suspension for 24 hours. This is the most chemically and mechanically intensive method of microfossil isolation but may be useful when sediments include relatively coarse-grained inorganic and organic matter.

A total of 200 cyanobacterial microfossils were enumerated for each replicate sample using Olympus BX41 at 400× magnification to ensure the evenness and comparability across the methods. Time taken for counting 200 microfossils was noted for each replicate and method. Samples were enumerated randomly. Taxonomic identification of fossils was based on John et al. (2002) and Shumilovskikh and Van Geel (2020) to the lowest feasible taxonomic level, including *Anabaena*, *Aphanizomenon* and *Gloeotrichia* spp. Total concentration for microfossils (microfossils mL^{-1}) was calculated following Bunting (2005) and Bunting et al. (2007).

Comparative difference in cyanobacterial microfossil recovery between different methods was identified using a Chi-square (χ^2) test. Differences in individual pairs of treatments were contrasted using a *t*-test. Box plots of cyanobacterial microfossil concentrations (microfossils mL^{-1}) and relative assemblage composition plot were used for visualising variability across methods.

Results

Marked differences in counting effort, microfossil recovery efficiency, and assemblage composition were identified across the four preparation methods. The impact of acetolysis was more pronounced than that of sieving in both lakes. Acetolysis-treated samples (M3 and M4) produced cleaner microscopic slides (fewer visual obstructions) than samples prepared without acetolysis (M1 and M2) but required considerably more time to reach 200 microfossils (Fig. 3). In Lough Aghery, time required to reach the standardised count increased from 22 min (M1) to 24 min (M2) and from 57 min (M3) to 120 min (M4), respectively (Table 1). Similarly, counting time for the Ballysaggart Lough samples increased from 32 min (M1) to 34 min (M2) and 157 min (M3) to 202 min (M4).

Table 1

Mean total akinete concentration (remains mL^{-1}) and time required (minutes) to count 200 cyanobacterial microfossils for each preparation method in Lough Aghery and Ballysaggart Lough.

Lake	Sieving	Acetolysis	Method	Mean total akinete concentration (microfossils mL^{-1})	Mean counting time (min)
Lough Aghery	-	-	M1	3,548,526	22
	+	-	M2	1790175	24
	-	+	M3	364767	57
	+	+	M4	167918	120
Ballysaggart Lough	-	-	M1	1133778	32
	+	-	M2	741076	34
	-	+	M3	88729	157
	+	+	M4	128803	202

The highest concentrations of microfossils were generally recovered using M1 and M2, whereas densities exhibited a considerable decline following the acetolysis treatment (M3 and M4) (Fig. 4). Across all treatments, highest microfossil concentrations were consistently yielded in M1 samples from both lakes. For both sites, samples exhibited approximately 90% decline in akinete concentrations following acetolysis (M3 and M4) when compared to non-acetolysis treatments. Sieved preparations generally yielded lower microfossil concentrations than their non-sieved counterparts (Table 1). Pairwise t-tests showed that microfossil densities were significantly different ($p \leq 0.05$) across all treatment comparisons in both lakes, except comparisons of M3 and M4 which did not differ in either lake ($p > 0.50$).

In Lough Aghery and Ballysaggart Lough, assemblage composition showed a pronounced reduction in relative abundance of microfossils from *Aphanizomenon* spp. in acetolysis-based treatment (M3 and M4), resulting in an over-estimation of the proportional abundance of *Anabaena* and *Gloeotrichia* (Fig. 5). However, there were differences among lakes in the misrepresentation of sub-dominant taxa. For example, *Gloeotrichia* showed a greater over-representation in chemically treated samples from Ballysaggart Lough relative to changes recorded in Lough Aghery. Pearson's chi-squared tests indicated

significant difference in assemblage composition among preparation methods in both Lough Aghery (χ^2 = 1011.7, df = 22, p < 0.001) and Ballysaggart Lough χ^2 = 720.74, df = 22, p < 0.001.

Discussion

This study demonstrates that the choice of sediment preparation method can substantially impact the recovery of cyanobacterial microfossils, including counting efficiency, total microfossil densities, and estimation of assemblage composition. Among all four preparation methods, acetolysis had the most pronounced effect, with a substantial reduction in microfossil recovery. Relative to M1, the least intensive method, acetolysis treatment reduced the total akinete concentration by 85–95% in both lakes, despite the differences in relative abundance of genera in both lakes. We conclude that cyanobacterial microfossils are susceptible to chemical degradation during acetolysis. While acetolysis is commonly used in palynological and non-pollen palynomorph preparations (Limaye et al. 2007) to improve the visibility of cyanobacterial remains, this advantage appears to be offset by selective loss of *Aphanizomenon* spp. and a two-fold or greater increase in counting effort.

Along with reduction in absolute microfossil abundance, changes in the assemblage composition are arguably of greater concern as they could bias interpretations of biogeochemical processes or ecological responses. As shown by chi-square analysis, these significant differences in the assemblage composition demonstrate taxon-specific variation in cyanobacterial recovery with more chemically intensive preparation methods. Further, these changes represent methodological artefacts rather than actual changes in community structure that can alter palaeoecological interpretations. For example, underestimation of diazotrophic *Aphanizomenon* spp., a taxon common to highly-eutrophied ecosystems (Bunting et al. 2016; Gushulak et al. 2024; Hayes et al. 2019), relative to *Gloeotrichia* spp., a genus common in early stages of fertilization (Ewing et al. 2020; Bunting et al. 2007), would underestimate the degree of damage by nutrient pollution. In addition, previous studies have associated *Anabaena* spp., with nitrogen limitation and water column stability, while *Aphanizomenon* spp. have been linked to reduced water transparency or N limitation, and *Gloeotrichia* spp. with warm, shallow, high-light conditions (Kling 2005). As a result, preparation-induced changes in the relative abundance of these taxa may influence reconstructions of historical nutrient dynamics, light availability, and habitat conditions. Similarly, underestimation of total akinete densities could be misinterpreted as low bloom intensity in the past, thereby delaying effective management actions or mis-identifying causal mechanisms underlying water quality degradation (e.g., onset of urban pollution).

Unlike acetolysis, the effect of sieving was less pronounced and consistent between lakes. Overall, sieved preparations generally reduced microfossil recovery relative to unsieved samples. Although the effect was small relative to that observed for acetolysis, differences were statistically significant and would be expected to underestimate the intensity of past cyanobacterial outbreaks. As previous studies have employed relative abundance of cyanobacterial taxa for inferring ecological responses to changes in nutrient status, hydrology or lake productivity (Bunting et al. 2007; Bunting et al. 2016; McCarthy et al. 2021; Van Geel et al. 1994), effects of sieving and acetolysis together seem likely to result in substantial

underestimation of reconstructed cyanobacterial abundance, misinterpretation of historical bloom magnitude, and distortion of past planktonic community composition. These findings further imply that direct comparison between published studies may be problematic when different preparation protocols have been applied, as observed differences in assemblage and abundance may reflect methodological artefacts rather than underlying ecological or limnological conditions.

Further research is required to evaluate why biases in community composition differed among study lakes. In general, underestimation of *Aphanizomenon* spp. densities was greater in Lough Aghery than in Ballysaggart Lough. In principle, differences in limnological features (deepwater anoxia, grazer composition, water-column depth, etc.) or the sedimentary preservation environment (chironomids, mineral composition, oxidized microzone, etc.) may alter the degree of microfossil degradation during sinking and consequently the effects of chemical treatment protocols. Alternately, different species of the same genus may vary in their susceptibility to acetolysis, leading to observed variation in underestimation of *Aphanizomenon* (or over-estimation of other taxa). Regardless of the mechanism, our findings demonstrate that minimal chemical preparation produced both the least biased population estimates and the most time-efficient enumeration needed to achieve the desired robustness of historical population estimates (200 microfossils sample^{-1}). Collectively, these findings suggest that the simplest preparation method (M1) offers the most reliable recovery while requiring least counting effort, at least in these eutrophic lakes. Because acetolysis causes substantial loss in the microfossil abundance, altered assemblage composition, and increased counting effort, we do not recommend its use for future paleolimnological studies of past cyanobacterial populations based on microfossil analysis.

Declarations

Research Funding

This research is funded through the Natural Environment Research Council as part of the QUADRAT Doctoral Training Partnership (DTP) under project reference number NE/S007377/1 to AM and HR, and by a Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant RGPIN-2024-04761 to PRL.

Author Contribution

Study was designed by all authors. Sample preparation, enumeration, and statistical analysis was conducted by AM. First draft and figures were produced by AM. All authors edited the manuscript and approved the final version.

Acknowledgement

We thank Jim Slon (School of Natural and Built Environment, Queen's University, Belfast) for technical and laboratory work assistance. We thank Professor Gill Plunkett for providing access to laboratory facilities and support with acetolysis preparation. We acknowledge Dr. Alastair Ruffell, Dr. Stephen Prentice (Northern Ireland Environmental Agency), and Ross Purvis with fieldwork and sediment sampling. We thank Cale Gushulak for comments on an earlier version of this paper. This research is funded through the QUADRAT doctoral training partnership to AM and HR, and by a Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant to PRL.

Data Availability

Data are available upon request.

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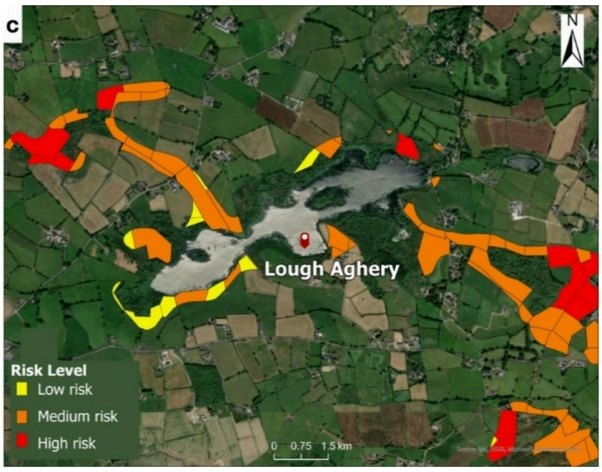
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Figures





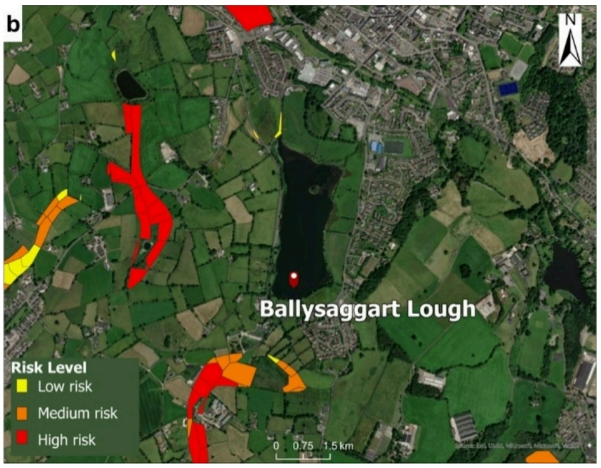


Figure 1

a) Location of Lough Aghery and Ballysaggart Lough and their respective catchments within Northern Ireland, UK. Enlarged map of (b) Ballysaggart Lough and (c) Lough Aghery with agricultural critical risk areas represent land modelled according to the likelihood of runoff entering water systems. (Source: OpenDataNI; map created in ArcGIS Pro.)

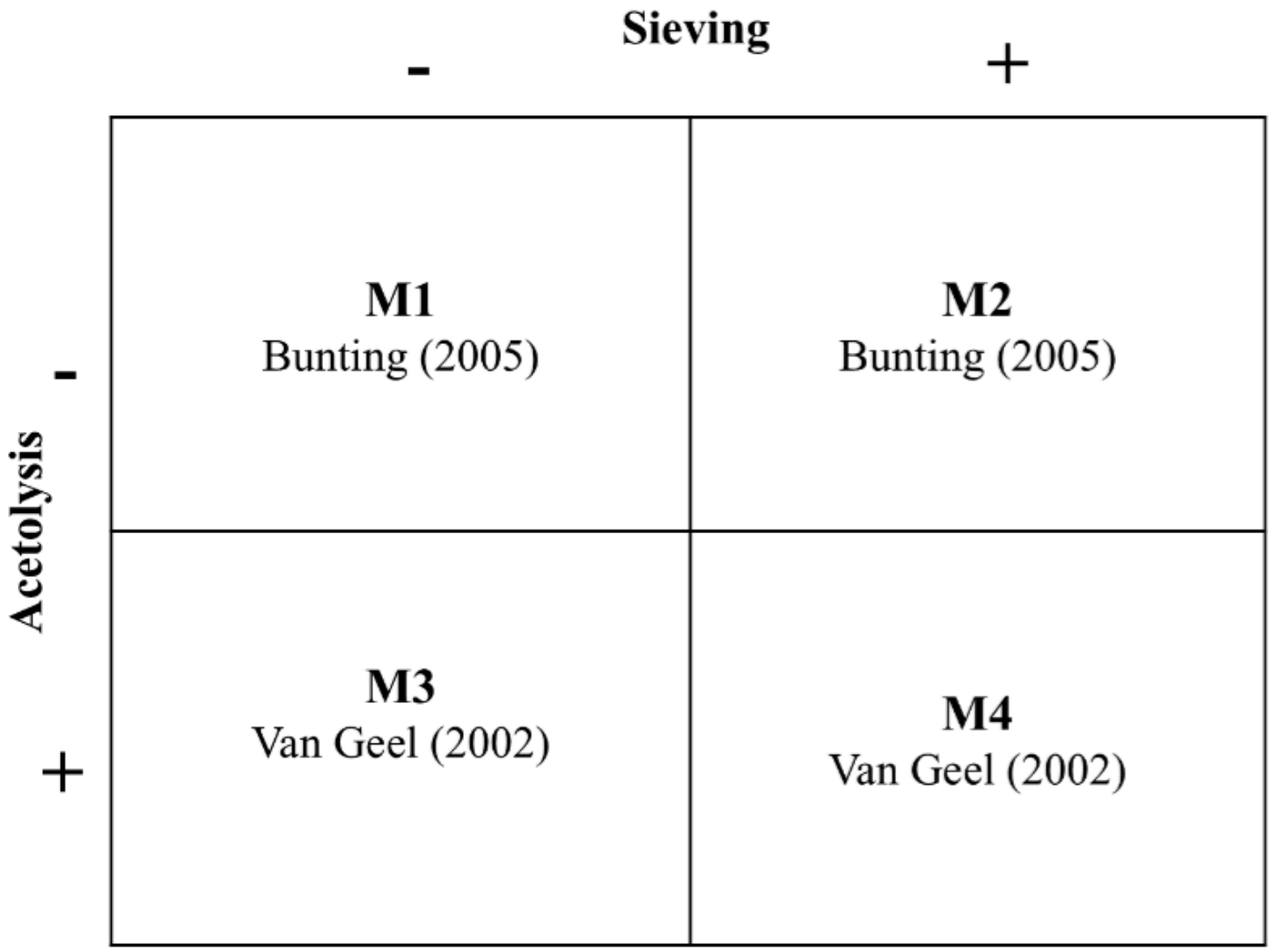


Figure 2

Schematic representation of the four sediment preparation methods used for cyanobacterial microfossil analysis. M1 = no sieving and no acetolysis; M2 = sieving only; M3 = acetolysis only; M4 = sieving and acetolysis.

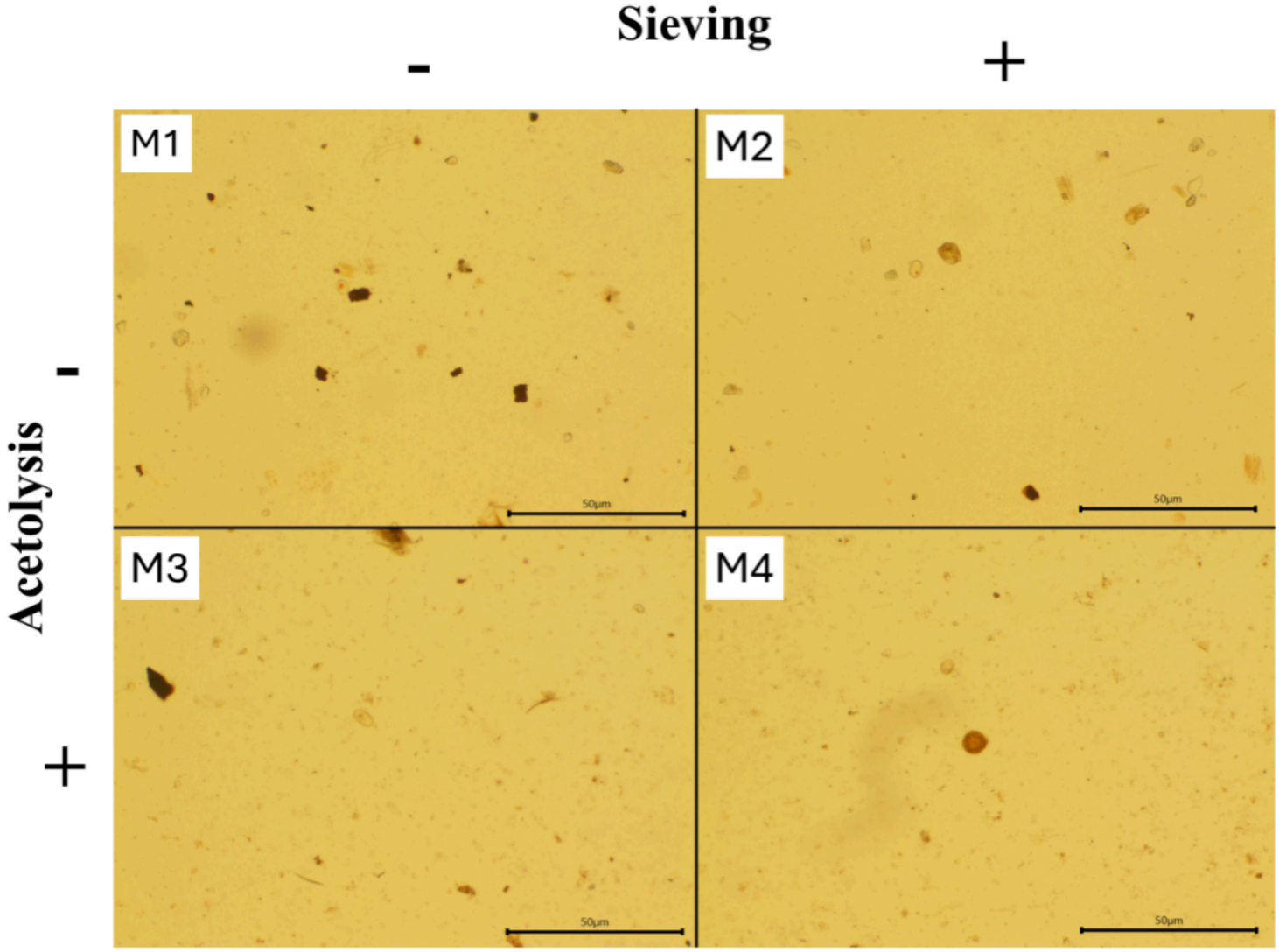


Figure 3

Representative microscope images of residues produced by the four preparation methods (M1–M4), illustrating differences in residue appearance and debris content after sample preparation. Scale bars = 50 µm.

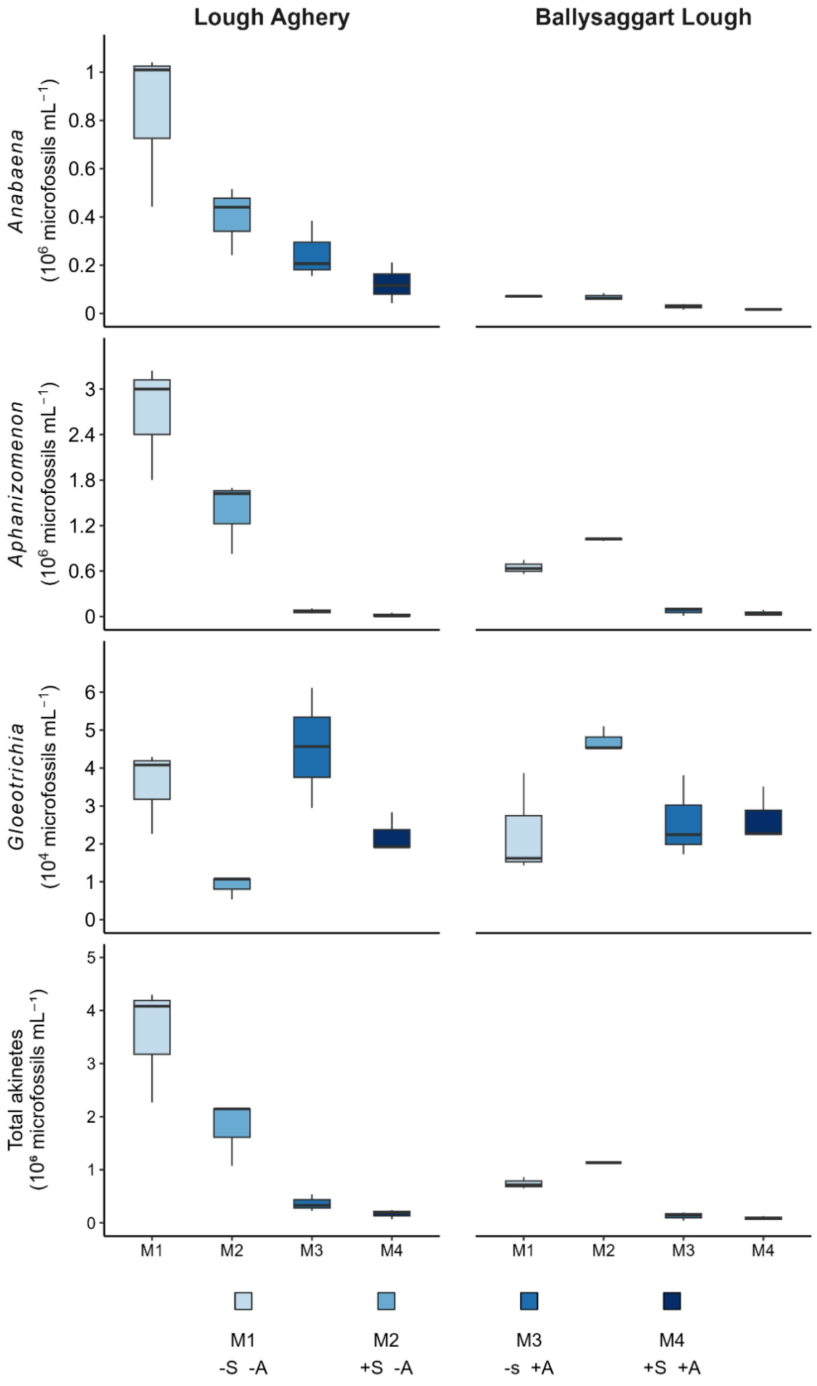


Figure 4

Concentrations of microfossils from *Anabaena, Aphanizomenon, Gloeotrichia* spp. and total akinetes recovered using four sediment preparation methods for samples from eutrophic Lough Aghery and Ballysaggart Lough.

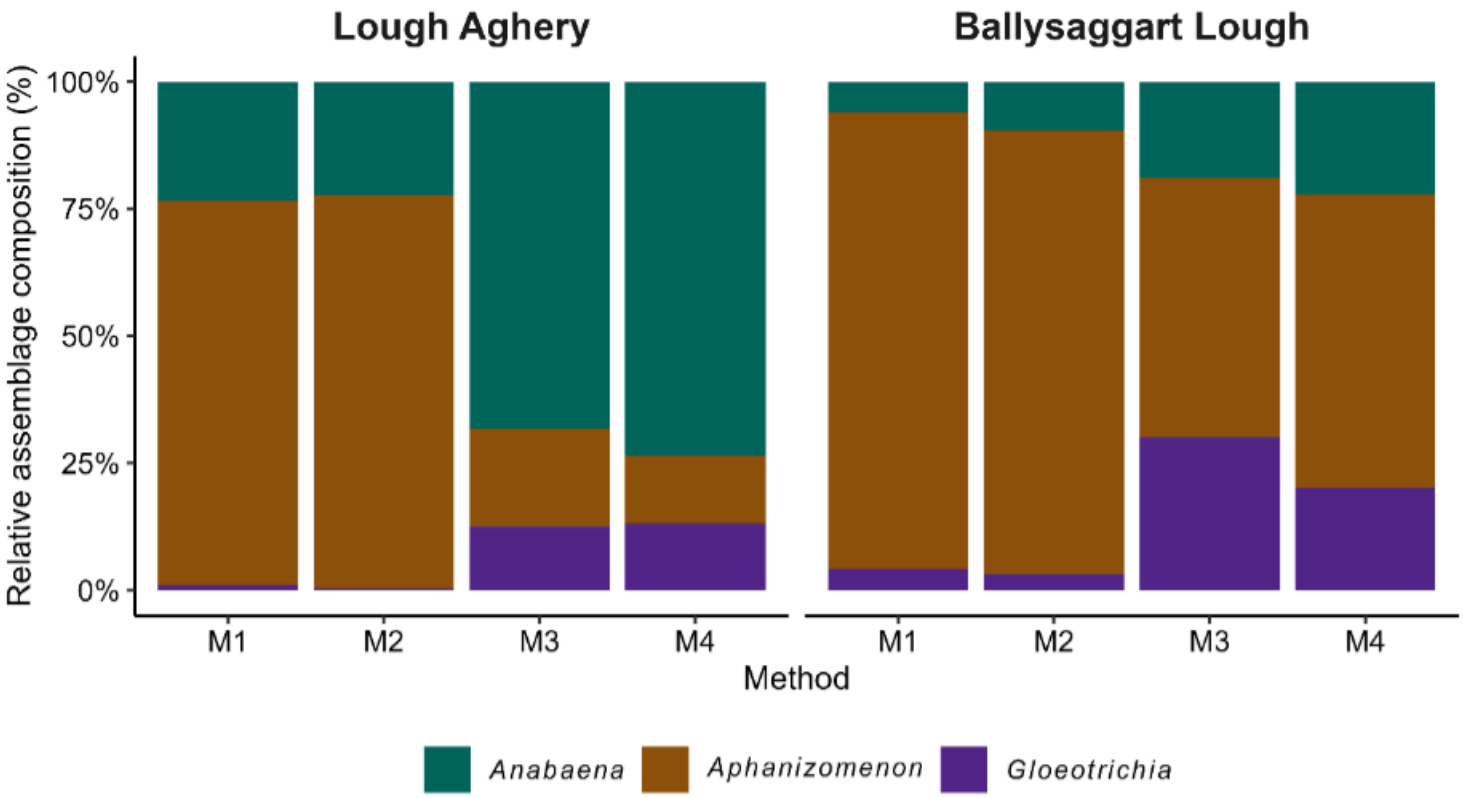


Figure 5

Relative assemblage composition of cyanobacterial microfossils recovered using the four sediment preparation methods from Lough Aghery and Ballysaggart Lough.

Supplementary Files

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- Flowchart.tif